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Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections

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14. ABSTRACT
In the fourth year, the in vivo efficacy of CHD-FA to treat cutaneous and burn wounds infected with multidrug resistant Gram-negative bacteria *P. aeruginosa* or Gram-positive bacteria MRSA was evaluated. In these trials, histopathological analyses and wound healing gene expression profiling demonstrated alleviated inflammation and promoted healing upon CHD-FA treatment. The bacterial burden assessment also found a significant burden reduction from wounds treated with CHD-FA. In both cutaneous and burn model of the 6-day *P. aeruginosa* study, bacteria were completely eliminated from infected wounds as rapidly as 24h post-inoculation with twice daily application of CHD-FA. Similarly, promising *in vivo* antimicrobial efficacy was also observed in the cutaneous and burn wound model with MRSA. However, the microbial burden restored from day 3 till day 6 in the cutaneous wound infection model. Although we have previously confirmed the broad-spectrum activity of CHD-FA *in vitro*, CHD-FA may be less active against Gram-positive pathogens *in vivo*. The exact molecular mechanisms of the antibiotic activity of CHD-FA are still not clear, and will be further investigated to address the discrepancy in its activity against Gram-positive and Gram-negative pathogens in our future work. By far, CHD-FA with the modified application method enhanced absorption of drugs through the eschar and is the most promising combination to control the infection caused by rapidly growing bacterial species. Overall, we have made significant progress in the fourth year in demonstrating the value of CHD-FA to treat wound infections. We believe that the CHD-FA combined with the modified application method will be effective in treating wounds infected with major multi drug resistant pathogens and demonstrate its universality for preventing wound infections and promote healing.

15. SUBJECT TERMS
carbohydrate-derived fulvic acid (CHD-FA), drug resistant wound infections,

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Introduction

The objective of this study is to demonstrate the potent antimicrobial properties of carbohydrate-derived fulvic acid (CHD-FA) against a broad collection of drug-sensitive and multi-drug-resistant (MDR) pathogens commonly associated with wound infections, and assess the relative efficacy of CHD-FA against induced wound infections in *in vivo* animal models. Overall, this work is intended to establish CHD-FA as a safe and effective agent that can be deployed to prevent the onset of drug-resistant bacterial and fungal infections in military and civilian personnel suffering traumatic wound infections. Given its novel mechanism of action and preliminary activity against MDR bacteria and antifungal-resistant fungi, the early use of CHD-FA is advantageous because it represents a novel target that will not select for resistant organisms, and prevents the use of more specific but more limited spectrum antibiotics. The overall goal of this preclinical program is to establish a firm justification for progressing to human trials to determine the efficacy of topical CHD-FA in preventing wound infections in injured military and civilian personnel.

Body

Section 2.1 Description of Overall Progress in the First Annual Report. (Sept 2012 to Sept 2013).

As described in the Annual Technical Report submitted on 10/29/2013, Specific Aim 1 of the statement of work establishing the *in vitro* efficacy of CHD-FA against drug resistant bacteria and fungi was completed. In the first year of this research contract we demonstrated the antimicrobial properties of Carbohydrate-Derived Fulvic Acid (CHD-FA) against a broad collection of multi-drug resistant bacterial and fungal pathogens commonly associated with wound infections. We completed the objectives in specific aim 1 of the award statement of work by determining the *in vitro* susceptibility of CHD-FA against a collection of multi-drug resistant *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, carbapenam-resistant *Klebsiella pneumoniae*, *Enterococcus faecium*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, Enterobacter spp., other clinically important species as well as azole resistant fungal pathogens *Candida* spp., *Aspergillus fumigatus* and polyene-resistant non-*fumigatus Aspergillus* species, *Fusarium* species and *Zygomycetes*. Most bacteria show complete growth inhibition at 0.06 – 0.125% fulvic acid, while efficacy with fungi is 0.125-0.5%. As stated in specific aim 2, small animal models were started during this period. We also established a cutaneous wound infection model in rats with MRSA and *Pseudomonas aeruginosa* to assess (CHD-FA) as a potent topical agent to prevent drug resistant wound infections and promote healing as part of specific aim 2 of the research contract. Daily topical applications of CHD-FA were placed on the cutaneous wounds in rats at 4 and 24h post infection for up to 10 days. Wound size and wound bacterial burdens were measured to assess the treatment efficacy of CHD-FA. CHD-FA treated animals had improved wound healing and reduced bacterial burden.

Section 2.2. **Description of Overall Progress in the Second Annual Report. (Sept 2013 to Sept 2014).**

As described in the Annual Technical Report submitted on 10/29/2014, in the second year, a cutaneous wound model in rats with the drug resistant Gram negative bacteria *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and pathogenic mold *Aspergillus fumigatus* was established. Efficacy of CHD-FA against cutaneous wounds infected with these organisms was assessed respectively. Significant wound surface area reduction with high and mid CHD-FA treatment doses was observed with all studied infection models. Remarkable bioburden reduction induced by CHD-FA was also observed in wounds infected with multidrug resistant *E. coli* and *K. pneumoniae*. To better assess wound healing upon CHD-FA treatment at cellular and molecular level, a more comprehensive histopathological evaluation and expression profiling of wound healing genes was performed at various time points during the infection/treatment course in rats infected with MRSA and *P. aeruginosa*, respectively. Histopathological analysis showed that the infection and inflammation in the wounds from both infection models treated with CHD-FA was better controlled relative to the untreated and antibiotic control groups, as indicated by lower neutrophils scores as early as day 3. The decrease in cellular inflammation and increase in epithelialization at the endpoint of the experiment suggested wound healing was significantly improved with CHD-FA treatment. Host gene expression profiling displayed the same dynamic trend of differentially expressed wound healing genes upon CHD-FA treatment in both infection models. The levels of overexpression were found to be more prominent in the CHD-FA group at day 3 compared to the untreated control, and their expression levels rapidly returned towards baseline at day 6, as the same set of genes in the untreated control increased. In particular, the key biomarker of impaired wound healing IL6 was constantly overexpressed day 3 to day 6 in the untreated wound. In contrast, expression of IL6 was significantly dampened at day 6 in the CHD-FA treated wounds, indicating an accelerated and better controlled wound healing.

A full preliminary evaluation of CHD-FA to treat wound infections with all the pathogens listed in the Statement of Work (SOW) was completed with the submission of this annual report. These preliminary results were encouraging and histopathological and host gene expression profiling on wounds infected will be performed with the other pathogens listed in the statement of work.

Section 2.3 Description of Overall Progress during the Third Annual Report. (Sept 2014 to Sept 2015).

As described in the Annual Technical Report submitted on 10/29/2015, in the third year, a burn wound model in rats was established and infection with *K. pneumoniae*, MRSA, *P. aeruginosa* and treated with CHD-FA were validated. Histopathological and host gene expression profiling analyses of burn wounds infected with *K. pneumoniae* and MRSA and treated with CHD-FA demonstrated improved healing at the cellular and molecular level. Specifically, CHD-FA treated wounds displayed decreased cellular inflammation, and increased rates of epithelialization. We performed the *in vivo* analysis of the newly formulated CHD-FA-Zn. Our initial results demonstrated that CHD-FA-Zn reduced microbial burdens of susceptible and drug-resistant planktonic bacteria, and significantly improved wound healing kinetics. Host expression profiling of wound healing genes showed similar behavior with CHD-FA treated wounds with a prominent effect of reducing inflammation at early stages of infection. Our studies also raised some open questions. For example, cutaneous wounds infected with MRSA and *P. aeruginosa*, and treated with CHD-FA-Zn had rapid microbial clearance until 48h post-inoculation. However, the microbial burdens restored from day 3 till day 6. The biofilm matrix formed on day 3 post-inoculation may be restricting the penetration of the drug. The newly formulated CHD-FA combined with the modified application method was proposed to be tested against infections caused by clinically important drug resistant pathogens in the no-cost extension year.

Section 2.4 Description of Overall Progress during the Current Annual Report. (Sept 2015 to Sept 2016).

During the last 4 quarters, we continued working on specific aim 2 of the statement of work and established cutaneous and burn wound model in rats with the drug resistant Gram negative bacteria *Pseudomonas aeruginosa*, and Gram positive Methicillin Resistant *Staphylococcus aureus*. We assessed the treatment efficacy of Carbohydrate-Derived Fulvic Acid (CHD-FA) combined with the modified application method against cutaneous and burn wounds infected with these organisms.

Section 3. Evaluation of CHD-FA-Zn to treat cutaneous wounds infected with Methicillin Resistant *Staphylococcus aureus* (MRSA) strain MW2 in 6-day study.

To assess the treatment efficacy of CHD-FA-Zn against cutaneous wounds infected with MRSA strain MW2, a total of 9 rats were randomized into three treatment groups: 3 in CHD-FA-Zn (batch#2815) group, 3 in PBS group, and 3 in Mupirocin group.

Methicillin Resistant *S. aureus* strain MW2 was inoculated in BHI media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to 10^4 CFU per ml for the infection. Two open wounds were created on each rat, for a total of 18 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from each treatment group were infected with 0.05 ml of the MRSA cell suspension to a final infection dose of 500 CFU.

Circular gauze dressing of 8mm in diameter was saturated overnight with either CHD-FA-Zn or PBS in sterile centrifuge tubes. These saturated dressings were applied to the wound site with sterile forceps twice daily starting at 30 min post inoculation**. Figure 2** shows representative images of the wounds over the course of day 0 (day of wound creation) through day 6.

Compared to PBS controls, less discharge was observed for wounds treated with CHD-FA-Zn and Mupirocin control throughout the 6 day study period. However, wound closure was not visually observed in the CHD-FA-Zn treated wounds (**Figure 2**). The bacterial burden assessment found a significant burden reduction from wounds treated with CHD-FA-Zn throughout the trial, in contrast to the steady increased burden with an average of 4.2 log CFU bacteria recovered from untreated wounds at 6 days post-inoculation (**Figure 1, table 1**.) However, a disturbing observation with CHD-FA-Zn was its ineffectiveness to eliminate the rapidly growing *Enterococcus sp.* contaminant (bacterial species was confirmed by 16S sequencing of the contaminant colonies) recovered from the wound on 6 days post-inoculation.

Conclusion

Multiple application of CHD-FA-Zn was not sufficient to control infections from other bacterial contaminants in a 6-day infection model. Furthermore, the zinc diacetate formulation of CHD-FA appears to be delaying wound closure and healing. We will evaluate effects of CHD-FA-Zn on the wound in the absence of infection in order to better assess the cellular and molecular mechanisms during wound healing.

Table 1. Bacterial burdens of cutaneous wounds infected with MRSA at Day 1,3, and 6 experimental endpoints.

MRSA 500CFU	Day 1			Day 3			Day 6			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	6.0	NA	NA	6.0	NA	NA	4.2	NA	NA	MRSA 500 CFU 0.5h Tx
CHD-FA wth Zinc Diacetate	Sterilized	NA	6.0	3.4	NA	2.7	Sterilized*	NA	4.2	
AB Control	Sterilized	NA	6.0	Sterilized	NA	6.0	2.3*	NA	1.9	

*Burdens of *Enterococcus sp*. is not shown on the table

Figure 1. Bacterial burdens of cutaneous wounds infected with MRSA vs. post-inoculation time.

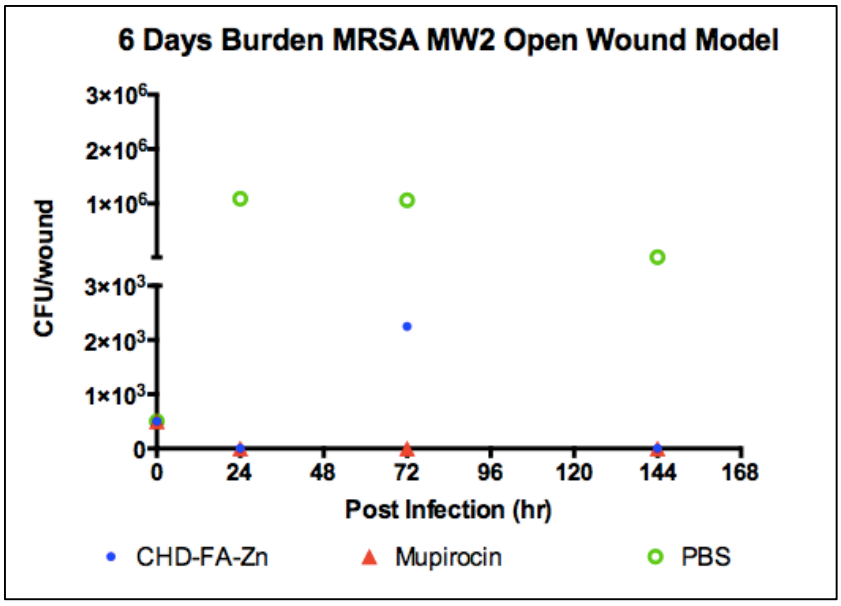
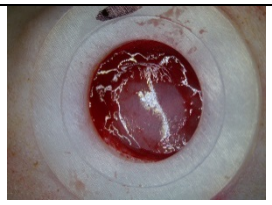
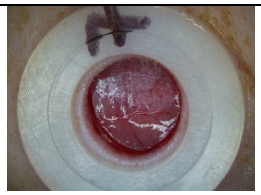
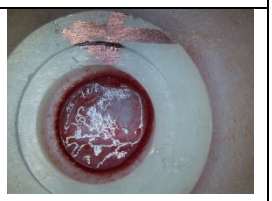
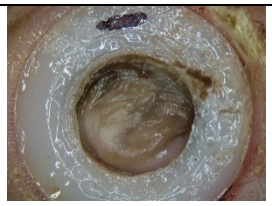
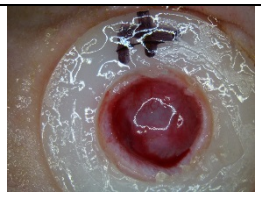
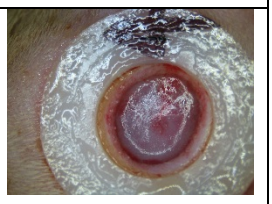
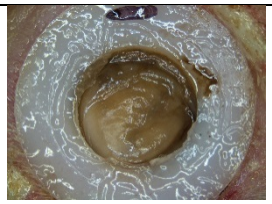
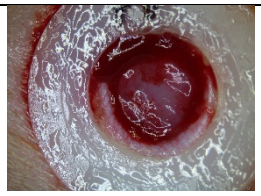
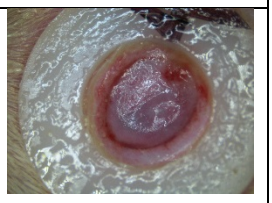
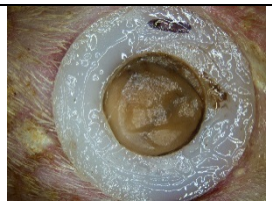
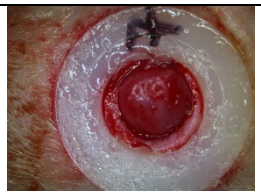
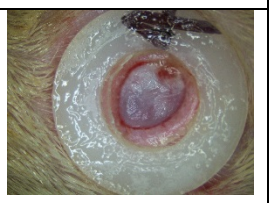
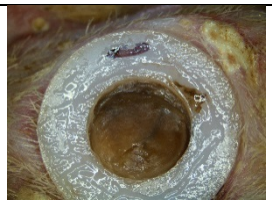
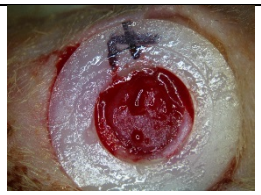
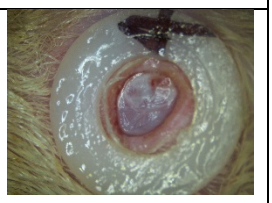
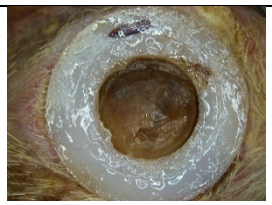
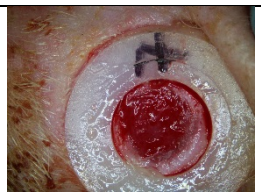
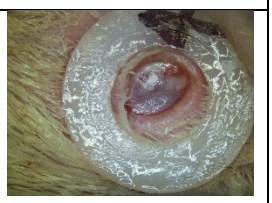
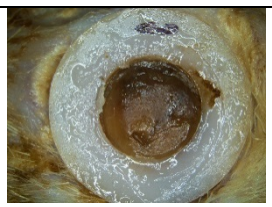
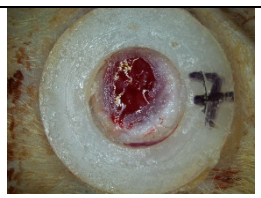
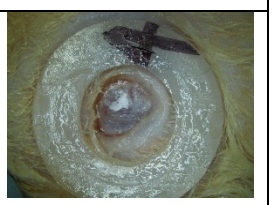


Figure 2. Cutaneous wound images of rats infected with 500 CFU of MRSA (MW2) and treated with CHD-FA-Zn at 0.5h post infection.

Day	CHD-FA with Zinc Diacetate	PBS	Mupirocin
0			
1			
2			
3			
4			
5			
6			

Section 4. Evaluating effectiveness of CHD-FA-Zn on cutaneous wound healing in the absence of infection.

To better understand the effects of CHD-FA-Zn on cutaneous wound healing in the absence of infection, we compared the healing process of cutaneous wounds treated with CHD-FA and vehicle by wound image, and clinical observation. A total of 3 rats were treated with CHD-FA and one rat was sacrificed at day 1, 3, or 6 post-inoculation time points. The process of anesthetizing the rats and creating the cutaneous wounds was the same as previously described.

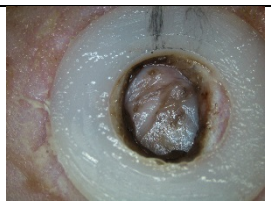
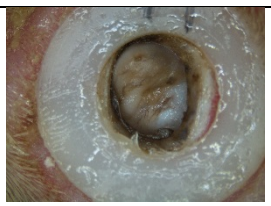
Circular gauze dressing of 8mm in diameter was saturated overnight with CHD-FA-Zn in sterile centrifuge tubes. This saturated dressing was applied to the wound site with sterile forceps twice daily starting at 30 min post inoculation**. Figure 3** shows representative images of the wounds over the course of day 0 (day of wound creation) through day 6.

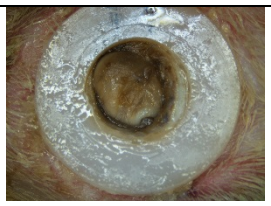
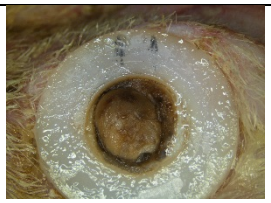
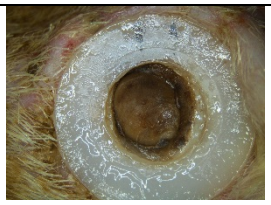
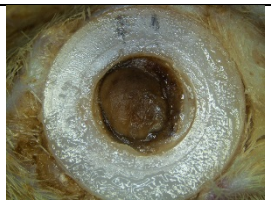
On day 3 post-treatment, one rat showed clinical symptoms of black ocular discharge, lethargy, and hunched posture. Other rats did not show such symptoms.

Conclusion

Multiple application of CHD-FA-Zn on the wound site appears to be toxic for the rat and the drug is also systemically delivered via the wounds. Although individual animal variance may have played a role, it is evident from the clinical observation that zinc formulation is causing an adverse systemic effect in rats. The zinc binding capacity of the porphyrin in rats may be responsible for carrying the zinc as the porphyrin is secreted, causing the black ocular discharge as described by Feng at al.

Figure 3. Images of cutaneous wounds treated with CHD-FA-Zn

Day	CHD-FA with Zinc Diacetate
0	
1	
2	

3	
4	
5	
6	

Section 5. Evaluation of modified application of CHD-FA to treat cutaneous wounds infected with *P. aeruginosa* Xen5.

Due to the observed toxicity of CHD-FA-Zn as mentioned above, we decided to evaluate the efficacy of CHD-FA gel (4.6%) formulation with the modified dressing application method. The cutaneous wound model infected with *P. aeruginosa* strain Xen5 was used for this evaluation. A total of 12 rats were randomized into three treatment groups: 6 in 4.6% CHD-FA gel group, 3 in PBS group, and 3 in Colistin group.

P. aeruginosa Xen5 was inoculated in LB media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to 10^4 CFU per ml for the infection. Two open wounds were created on each rat, for a total of 12 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from each treatment group were infected with 0.05 ml of the *P. aeruginosa* cell suspension to a final infection dose of 500 CFU. Rats were given corresponding treatments in 0.05 ml volumes starting at 30min post inoculation.

Circular gauze dressing of 8mm in diameter was saturated overnight with either CHD-FA, Colistin, or PBS in sterile centrifuge tubes. This saturated dressing of the corresponding treatments was applied to the wound site with sterile forceps twice daily starting at 30 min post inoculation**. Figure 4** shows representative images of the wounds over the course of day 0 (day of wound creation) through day 6.

Compared to PBS and Colistin treated wounds, less blood discharge was observed in wounds treated

with CHD-FA. Bacterial burden in the wounds treated with CHD-FA was significantly reduced by 5.2, 4.5, and 6.3 log relative to the untreated control at 1 day, 3 days, and 6 days post-inoculation, respectively. In comparison, Colistin treated wounds were sterilized at 24h post-inoculation, but quickly recovered with 7.1 and 7.6 logs of *P. aeruginosa* on day 3 and day 6, respectively (**Figure 4, Table 2**).

Wound healing gene expression profiling was performed with a commercial PCR array (The Rat Wound Healing RT² Profiler™ PCR Array, Cat No. 330231, Qiagen), as previously described. Expression profiles of wound healing genes were compared between samples treated with CHD-FA and negative control at days 1, 3, and 6 post-infection. Consistent with results reported previously (2nd Annual Technical Report 10.2014), we observed a panel of genes with increased expression including CCL12, CCL7, CSF2, CSF3, CXCL1, CXCL11, CXCL3, CXCL5, IL10, IL1b, IL6, MMP9, PTGS2, SERPINE1, and TNF; and decreased expression was found with genes ACTC1, CDH1, Col14a, ITGB6, and TGFA, from samples collected at both time points. In particular, CHD-FA treated wound had more dampened inflammatory chemokine CXCL3 and CXCL5 expression level at day 6 post-infection while these genes in the sham control gradually increased **(Figure 6 & 7.)**

In addition, the rapid restoration of differentially regulated expression level of wound healing genes in the CHD-FA treated wounds demonstrated a faster cellular response and tissue repair exerted by CHD-FA. Specifically, the expression level of IL6 (wound healing impairment) was gradually increased during the first 6 days of experiment and was greatly increased by >3524-fold at day 6 in the untreated wound. In contrast, in the CHD-FA treated wound, the expression level remained low (24, 55, 52-fold increase on day 1, 3, and 6, respectively) throughout the experiment **(Table 3.)** Based on this observation, prolonged overexpression of IL6 in the untreated wound is associated with impaired wound healing, whereas low expression level clearly demonstrated the accelerated wound healing CHD-FA treated wound.

Histopathological evaluation of wounds from each treatment group was also performed throughout the study period at days 1, 3, and 6 post-inoculation. The detailed description of the scoring system for histological analysis is listed on **Table 4**. The histologic evaluation of CHD-FA and untreated wounds from day 3 and day 6 showed higher score for both fibroblast, neovascularization, and epithelialization categories in the 4.6% CHD-FA treated wound (**Table 5.**) This demonstrates a more advanced wound healing with CHD-FA treatment. No significant difference on scores for the presence of neutrophils and macrophages between CHD-FA treated and untreated wounds was noted in this trial.

Conclusion

Application of CHD-FA in a dressing over the wound resulted in a rapid wound sterilization. Gene expression profiling, and histopathological examination of wound infected with *P. aeruginosa* demonstrated faster and better balanced inflammatory response and wound healing process upon CHD-FA application, consistent with previous observations in cutaneous wounds and burns. CHD-FA with the modified application method is by far the most promising combination to control the infection caused by rapidly growing bacterial species such as *P. aeruginosa*. The future experiments will be focused on analyzing efficacy of this specific application of the CHD-FA gel formulation on cutaneous and burn wounds infected with different pathogens.

Table 2. Bacterial burdens of cutaneous wounds infected with *P. aeruginosa* Xen5 at Day 1,3, and 6 experimental endpoints.

P. aeruginosa 500 CFU	Day 1			Day 3			Day 6			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	5.2	NA	NA	7.5	NA	NA	7.2	NA	NA	*P. aeruginosa* 500 CFU 0.5h Tx
CHD-FA	Sterilized	NA	5.2	3.0	1.7-4.2	4.5	0.9	0.0-1.7	6.3	
AB Control	Sterilized	NA	5.2	7.1	NA	0.4	7.6	NA	-0.4	

Figure 4. Bacterial burdens of cutaneous wounds infected with *P. aeruginosa* Xen5 vs. post-inoculation time.

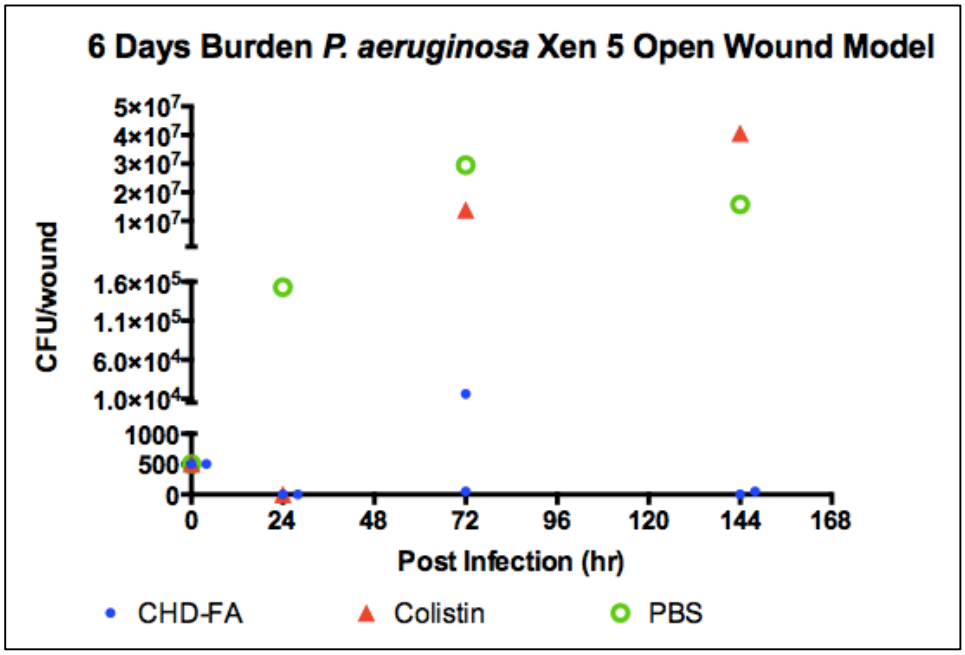
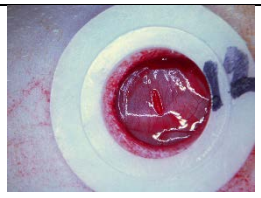
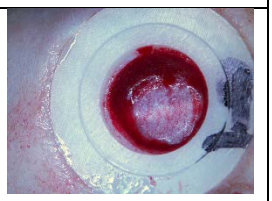
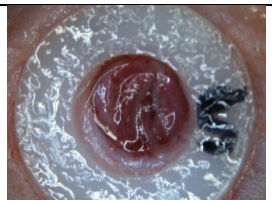
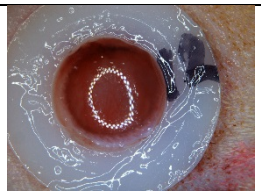
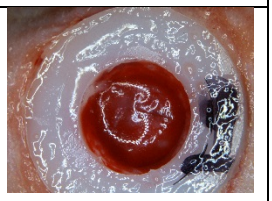


Figure 5. Cutaneous wound images of rats infected with 500 CFU of *P. aeruginosa* Xen5 and treated with CHD-FA at 0.5h post infection.

Day	CHD-FA	PBS	Colistin
0			
1			

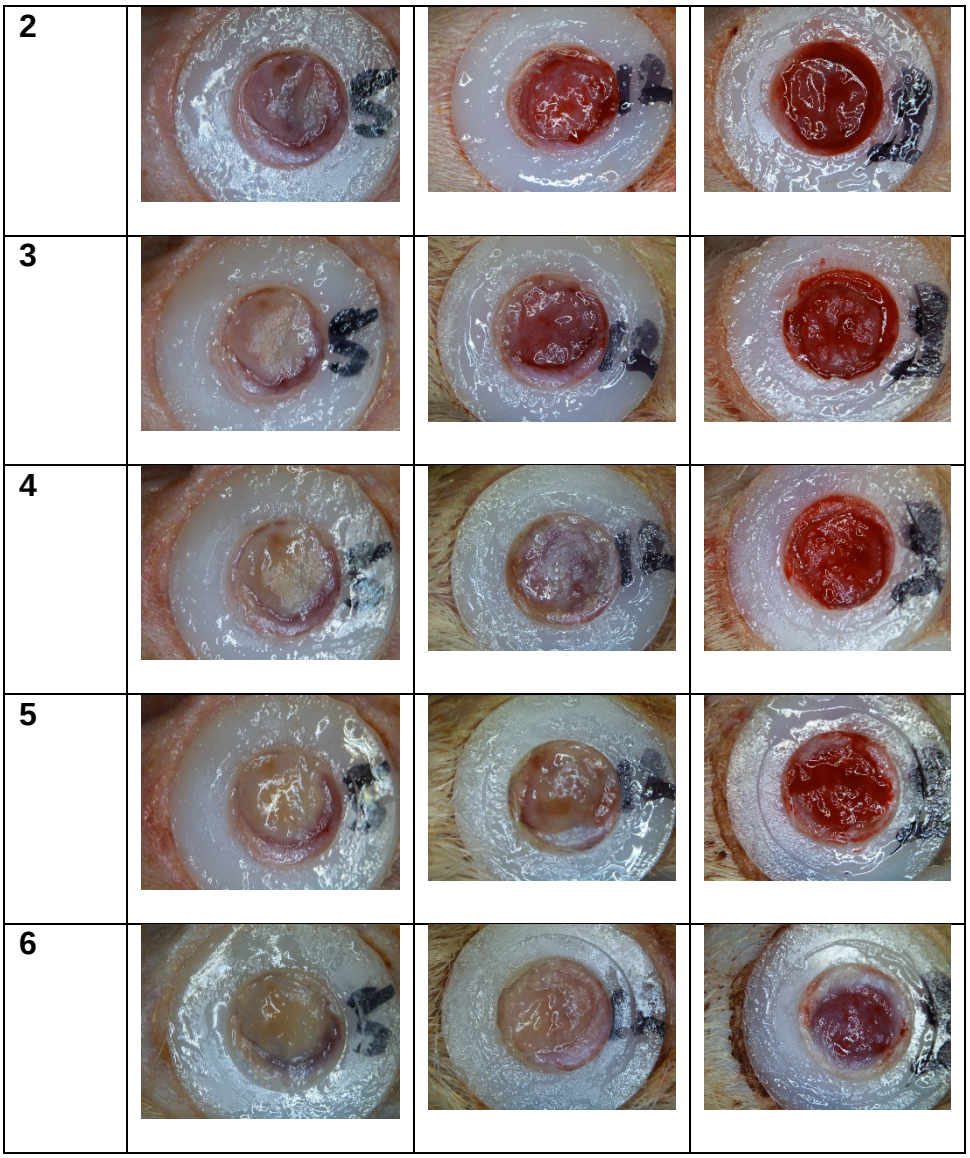
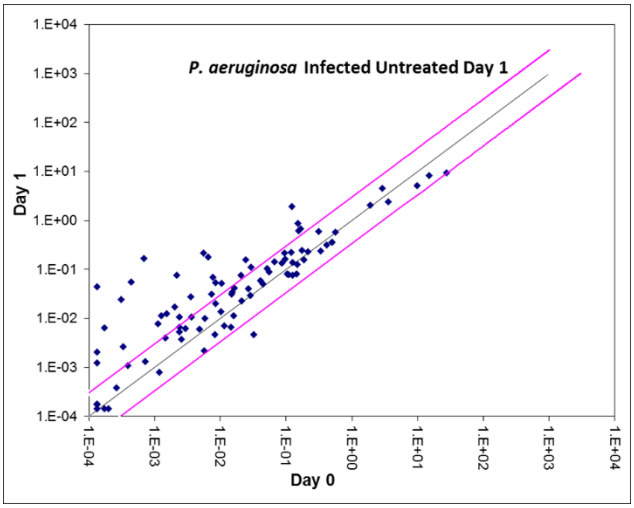
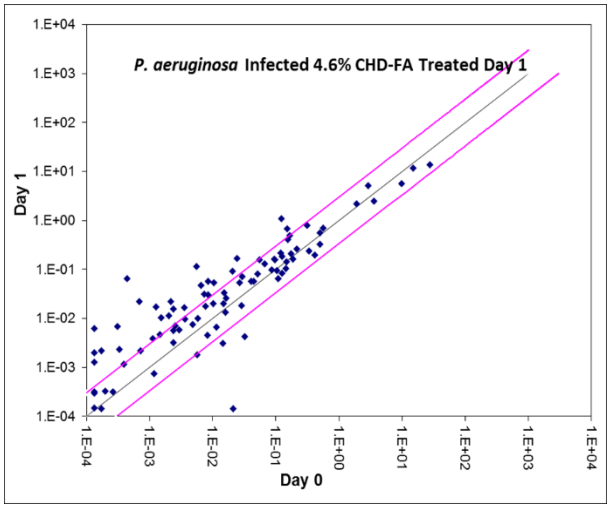


Figure 7. Scatter plot expression profiling of host wound healing genes along the study period at day 1, 3, and 6.





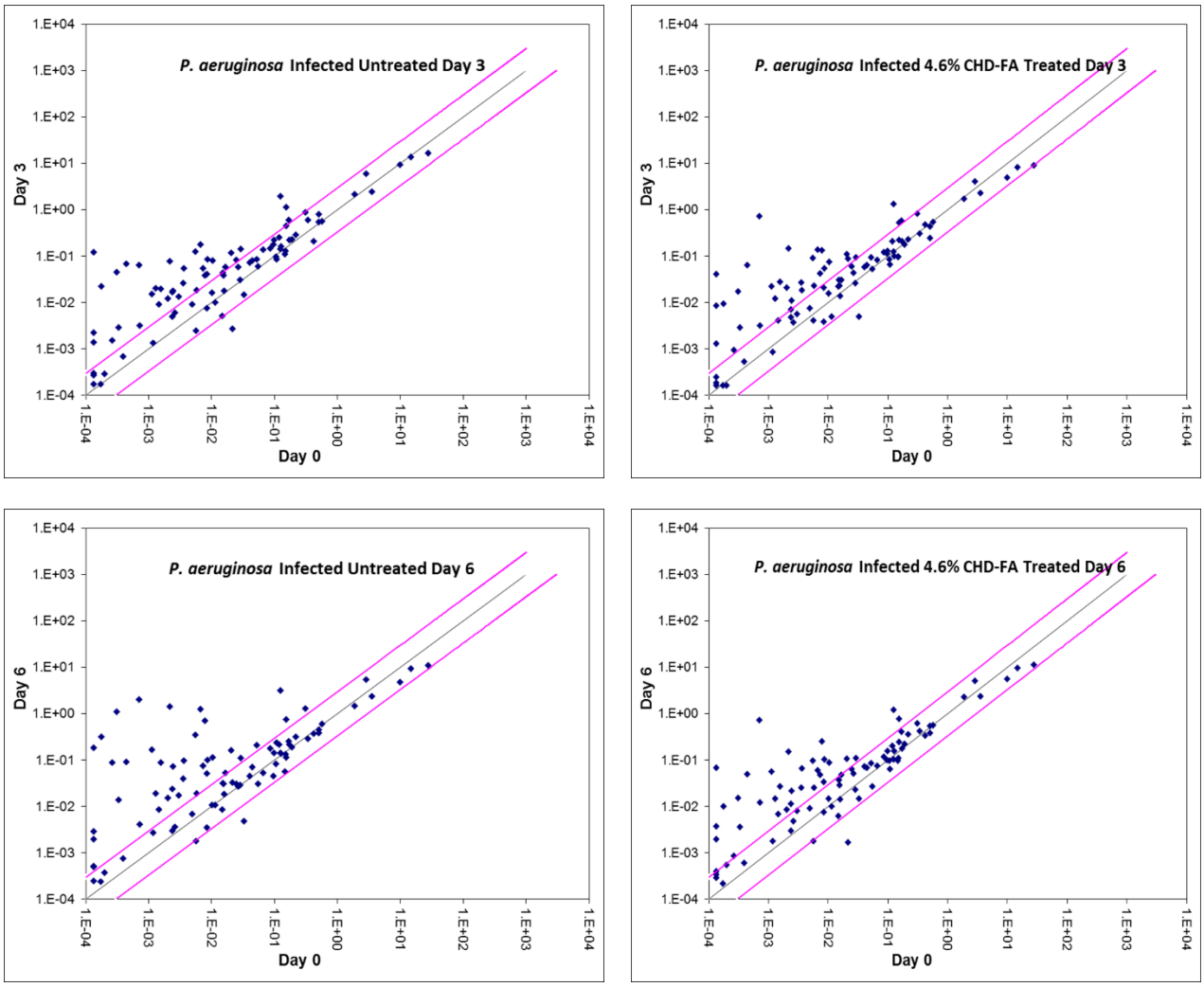
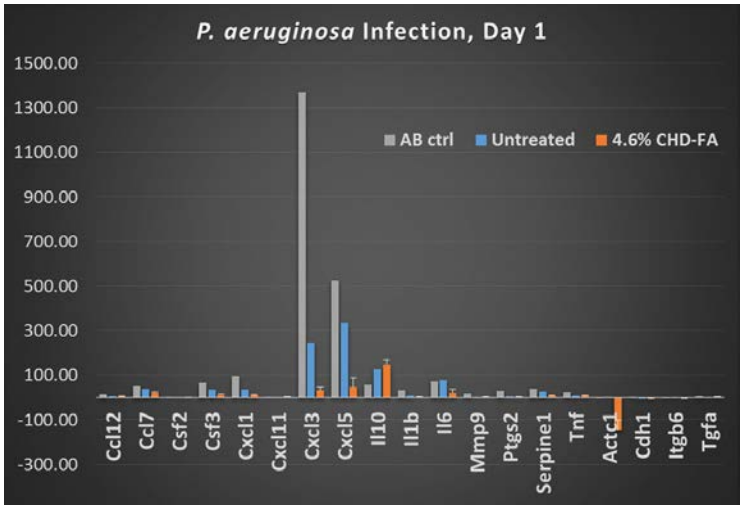
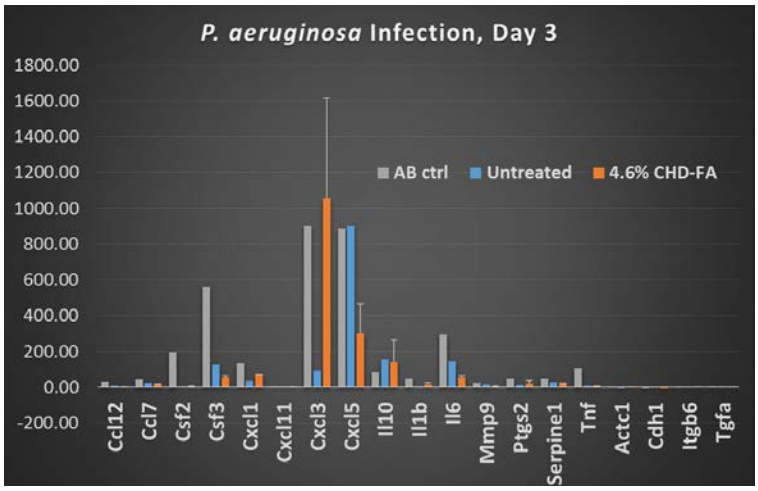


Figure 8. Fold changes of host wound healing genes treated with CHD-FA.





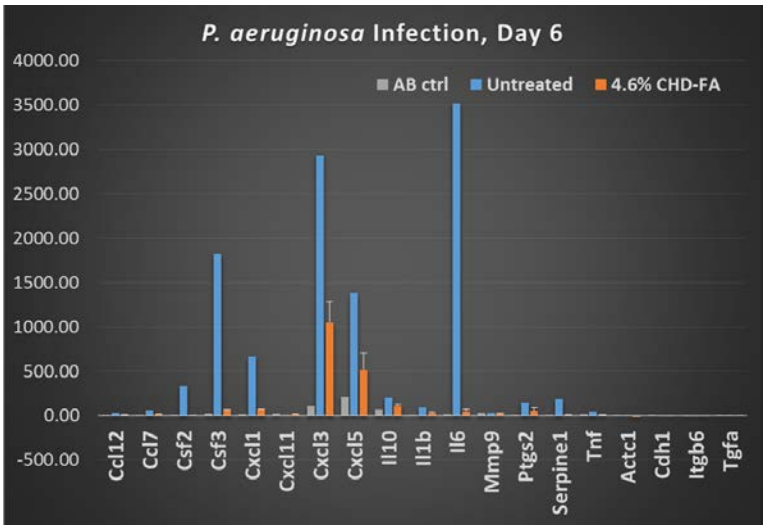


Table 3. Kinetic changes of prominently up-regulated wound healing genes among different treatment groups

Genes	AB Control				Untreated				4.6% CHD-FA			
	Day 0	Day 1	Day 3	Day 6	Day 0	Day 1	Day 3	Day 6	Day 0	Day 1	Day 3	Day 6
Ccl12	1	14.49	31.69	7.68	1	4.51	7.46	30.13	1	6.55	4.67	9.06
Ccl7	1	51.52	42.25	7.90	1	39.18	23.25	63.91	1	21.14	16.73	17.69
Csf2	1	2.90	194.82	2.97	1	1.45	5.81	337.33	1	1.21	3.81	3.23
Csf3	1	67.28	560.67	19.12	1	36.18	126.59	1824.14	1	12.92	55.14	58.47
Cxcl1	1	95.80	135.86	14.19	1	34.82	35.48	660.77	1	10.45	69.28	69.49
Cxcl11	1	1.84	3.26	22.66	1	1.80	4.36	5.69	1	3.01	4.46	17.12
Cxcl3	1	1367.19	901.39	105.20	1	245.06	93.31	2932.67	1	33.37	1124.95	1064.37
Cxcl5	1	525.30	885.90	210.40	1	334.76	900.14	1387.24	1	55.69	325.31	533.53

Il10	1	57.96	87.49	67.74	1	125.54	154.24	205.50	1	148.98	166.67	112.51
Il1b	1	31.28	45.92	10.18	1	8.77	5.01	90.07	1	2.75	18.39	32.66
Il6	1	70.86	296.32	11.21	1	77.01	146.93	3524.00	1	24.71	55.53	52.67
Mmp9	1	16.19	25.65	27.61	1	2.98	15.23	27.06	1	2.66	5.13	18.92
Ptgs2	1	28.29	46.88	7.00	1	6.88	13.54	148.36	1	3.51	23.51	58.10
Serpine1	1	39.18	48.03	5.11	1	26.95	26.80	187.14	1	7.38	20.74	9.35
Tnf	1	24.37	103.68	12.40	1	8.09	8.72	42.31	1	7.17	8.66	10.86

Table 4. Scoring summary for histological analysis

Score	Neutrophils	Macrophages	Fibroblasts	Angiogenesis	Epithelialization
0	No Neutrophils	No macrophages	No fibroblasts	None	No epithelialization
1	Scattered Neutrophils	Scattered macrophages	Poorly organized fibroblasts	Rare immature vessels	Early epithelialization at edges of wound
2	Multifocal aggregates of Neutrophils	Clusters of macrophages	Moderately organized fibroblasts	Moderate numbers of immature vessels	Complete epithelialization, but not all layers
3	Diffuse infiltrates of neutrophils	Diffuse infiltrates of macrophages	Well organized fibroblasts	Many immature vessels	Complete epithelialization

Table 5. Histopathological analysis of wounds from rats infected with *P. aeruginosa* Xen5 and treated with CHD-FA at 30m post infection.

	Day1		Day 3		Day 6	
4.6% CHD-FA	**Neutrophils**	**1**	**Neutrophils**	**2**	**Neutrophils**	**3**
	Macrophages	**1**	**Macrophages**	**2**	**Macrophages**	**3**
	Fibroblasts	**1**	**Fibroblasts**	**2**	**Fibroblasts**	**3**
	Angiogenesis	**1**	**Angiogenesis**	**2**	**Angiogenesis**	**3**
	Epithelization	**1**	**Epithelization**	**2**	**Epithelization**	**2**
	The wound lacked epidermis, dermis and some subcutaneous muscle with the underlying markedly inflamed subcuticular connective tissue and muscle filling much of the defect. A thin layer of the exposed and superficial subcutis was degenerate or necrotic with surface bacteria. Deeper subcutis contained large amount amounts of edema, fibrin, neutrophils, lymphocytes, macrophages and scattered fibroblasts, hemorrhage and congestion.		The well-demarcated wound lacked epidermis, dermis and subcutaneous muscle with the underlying subcuticular connective tissue extending into the defect. Exposed and superficial subcutis is expanded by extensive edema and dilated vasculature admixed with scattered bacteria. A thin granulation bed contained fibroblasts, lymphocytes, macrophages and increased numbers of blood vessels. There was marked expansion of the underlying deep subcutis by edematous inflammation.		The wound defect was replaced by a thick, bacteria-laden crust of neutrophilic (suppurative) necrotic debris over necrotic cell debris and a well demarcated bed of proliferating granulation tissue fibrocytes aligning parallel to the skin surface. Epithelium extended over the edges of the granulation tissue. The granulation bed was separated from the bacteria-laden crust by a zone of necrotic cellular debris.	
	Neutrophils	**1**	**Neutrophils**	**3**	**Neutrophils**	**1**

Antibiotic treated	**Macrophages**	**1**	**Macrophages**	**3**	**Macrophages**	**1**
	Fibroblasts	**1**	**Fibroblasts**	**3**	**Fibroblasts**	**1**
	Angiogenesis	**0**	**Angiogenesis**	**2**	**Angiogenesis**	**3**
	Epithelization	**0**	**Epithelization**	**2**	**Epithelization**	**2**
	There was a well-demarcated wound lacking epidermis, dermis and subcutaneous muscle with the underlying subcuticular connective tissue extending into the defect. Exposed and superficial subcutis was expanded by extensive edema and dilated vasculature. The adipocytes of the subcuticular fat were reduced in number and admixed with numerous bodies with scalloped margins, small black pigment specks and a birefringent-like quality. Remaining adipocytes were separated by edema, hemorrhage and leukocytes.		The well-demarcated wound lacked epidermis, dermis and subcutaneous muscle with the underlying markedly inflamed subcuticular connective tissue and filling much of the defect. Exposed hypodermis was covered by a thick mat of necrotic cell debris fibrin and degranulate neutrophils (pus) admixed with scattered bacteria and several birefringent, clear scalloped bodies. A prominent bed of granulation tissues was separated from the necrotic surface by a layer of fibrin admixed with edema, neutrophils and few fibroblasts and fibrocytes. The granulation bed contained fibroblasts, lymphocytes, macrophages and increased numbers of blood vessels.		The wound was replaced by a thick, proliferating granulation bed with fibrocytes aligning parallel to the skin surface. Epithelium extended over a small portion of the granulation tissue. The granulation bed surface contained small numbers of bacteria, hemorrhage, necrotic cell debris and neutrophils while the proliferating fibrovascular stroma extended into the deep subcutis.	
Untreated	**Neutrophils**	**1**	**Neutrophils**	**1**	**Neutrophils**	**3**
	Macrophages	**1**	**Macrophages**	**1**	**Macrophages**	**3**
	Fibroblasts	**1**	**Fibroblasts**	**1**	**Fibroblasts**	**3**
	Angiogenesis	**0**	**Angiogenesis**	**1**	**Angiogenesis**	**1**
	Epithelization	**0**	**Epithelization**	**1**	**Epithelization**	**0**
	The wound lacked epidermis, dermis and subcutaneous muscle with the underlying subcuticular connective tissue extending slightly into the defect. The exposed and superficial subcutis contained small amounts of hemorrhage, fibrin, neutrophils, and lymphocytes.		There was a well-demarcated wound lacking epidermis, dermis and subcutaneous muscle with underlying markedly inflamed subcuticular connective tissue that filled much of the defect. Exposed hypodermis was composed of necrotic cell debris, rare bacteria and several birefringent clear scalloped bodies. There was deeper accumulations of fibrin admixed with cell debris, lymphocytes, fibroblasts, collagen and blood vessels. The panniculus was expanded by increased numbers of fibroblasts, lymphocytes, and macrophages. The margins of the defect contained similar inflammation.		The wound area and most of the adjacent haired skin was necrotic with the dermis and subcutis expanded by neutrophilic debris (pus), inflammation and bacteria. The wound surface and margins were composed of necrotic, bacteria-laden pus that extended the length of the subcutis (under the wound margins).	

Section 6. Effects of iron on the antimicrobial properties of CHD-FA

This experiment was set to test the hypothesized mode of action of CHD-FA, in which CHD-FA deprives bacteria of the iron essential for growth and hence impairs the ability of bacteria to survive in the host during the infection. MIC testing was performed for CHD-FA against MRSA strain MW2 and *P. aeruginosa* strain Xen5 in the presence of $FeCl_3$.

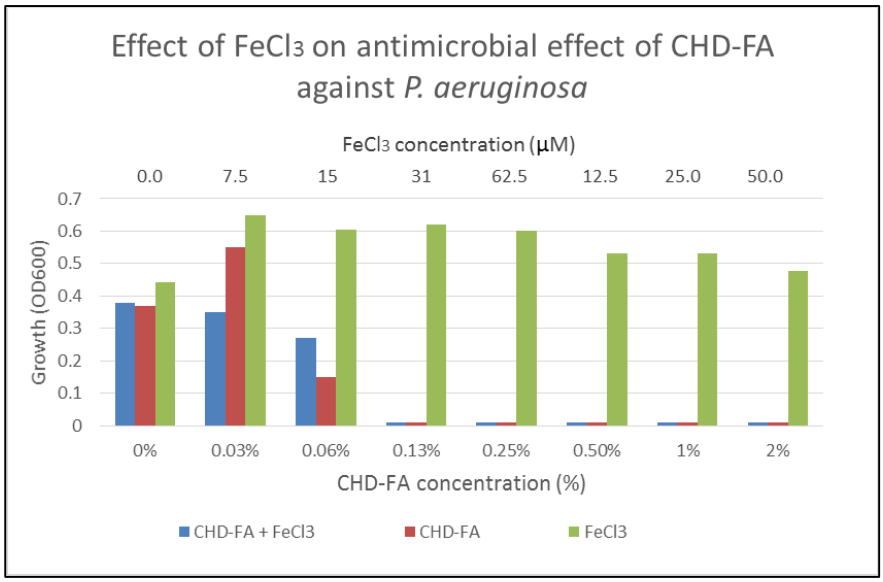
CHD-FA was supplied as a 4.6% Gel (Aug 2014 batch) by Fulvimed (Pty) Ltd (A division of Fulhold Ltd.), Somerset West, South Africa. CHD-FA was stored in the dark at room temperature. A highly standardized broth-based *in vitro* susceptibility assay following the Clinical and Laboratory Standards Institute (CLSI) protocol M07-A9 “Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Eighth Edition” was used to determine the MIC values. Briefly, bacterial colonies from a freshly grown agar plate were re-suspended in CLSI recommended cation-adjusted Mueller Hinton broth for aerobic bacteria. MIC values were visually read at 48h. The

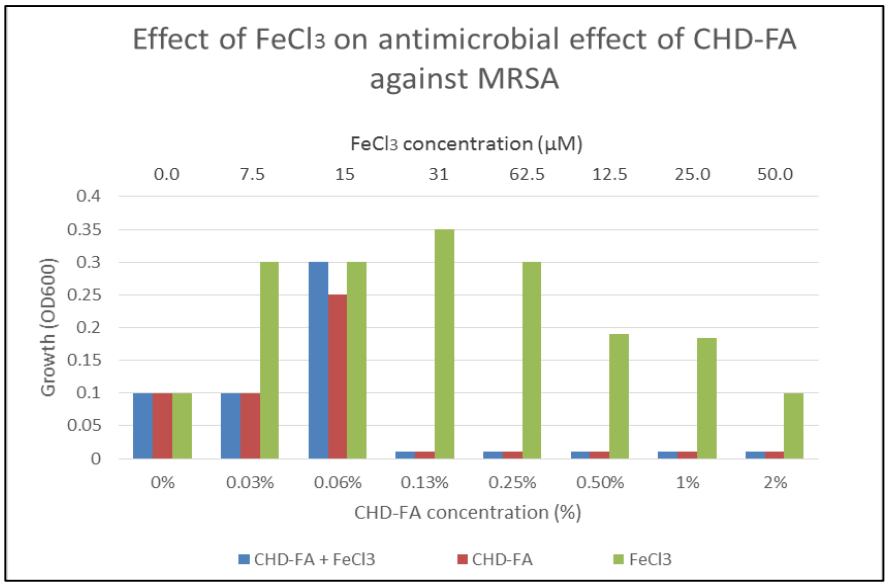
MIC is defined as the lowest concentration producing a prominent decrease in turbidity compared to the drug free well. All assays were performed in duplicate.

The 4.6% CHD-FA gel MIC against *P. aeruginosa* and MRSA is 0.13% in the presence of $FeCl_3$ and in the absence of $FeCl_3$ at 48h. $FeCl_3$ alone failed to inhibit growth of either pathogens.

Overall, there was no significant change in the MIC against *P. aeruginosa* or MRSA treated with CHD-FA in the presence of $FeCl_3$. These preliminary results demonstrate that there is little correlation between iron-limiting conditions and impaired survival of bacteria (See **Figure 6**.)

Figure 6. MIC of *P. aeruginosa* Xen5 and MRSA MW2 to CHD-FA in the presence of $FeCl_3$





Section 7. Evaluation of modified application of CHD-FA to treat cutaneous wounds infected with MRSA strain MW2.

To assess the treatment efficacy of CHD-FA against cutaneous wounds infected with MRSA strain MW2, a total of 12 rats were randomized into three treatment groups: 6 in CHD-FA (4.6%) group, 3 in PBS group, and 3 in Mupirocin group.

MRSA strain MW2 was inoculated in BHI media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to 10^4 CFU per ml for the infection. Two open wounds were created on each rat, for a total of 24 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from each treatment group were infected with 0.05 ml of the MRSA cell suspension to a final infection dose of 500 CFU.

Circular gauze dressing of 8mm in diameter was saturated overnight with either CHD-FA or PBS in sterile centrifuge tubes. These saturated dressings were applied to the wound site with sterile forceps twice daily starting at 30 min post inoculation**. Figure 8** shows representative images of the wounds over the course of day 0 (day of wound creation) through day 6.

Compared to PBS and Mupirocin treated wounds, less blood discharge was observed in wounds treated with CHD-FA. Bacterial burden in the wounds treated with CHD-FA was significantly reduced by 2.0, 1.8, and 5.4 log relative to the untreated control at 1 day, 3 days, and 6 days post-inoculation, respectively. In comparison, Mupirocin treated wounds were sterilized throughout the 6-day experiment. (**Figure 7, Table 6**.).

Conclusion

Similar to what observed in the *P. aeruginosa* infection model, CHD-FA treated wounds had a significant bacterial burden reduction, whereas untreated wound burden remained high over the course of the 6-day experiment. We will perform host gene expression profiling of the wounds, and histopathological analyses to better assess the host-pathogen-drug interaction. The future experiments will be focused on analyzing efficacy of this specific application of the CHD-FA gel formulation on cutaneous and burn wounds infected with different pathogens.

Table 6. Bacterial burdens of cutaneous wounds infected with MRSA at Day 1,3, and 6 experimental endpoints.

MRSA 500CFU	Day 1			Day 3			Day 6			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	5.9	NA	NA	6.4	NA	NA	7.1	NA	NA	MRSA 500 CFU 0.5h Tx
CHD-FA	3.8	3.7-3.9	2.0	4.6	4.2-5.0	1.8	1.7	0.0-3.4	5.4	
AB Control	Sterilized	NA	5.9	Sterilized	NA	6.4	Sterilized	NA	7.1	

Figure 7. Bacterial burdens of cutaneous wounds infected with MRSA vs. post-inoculation time.

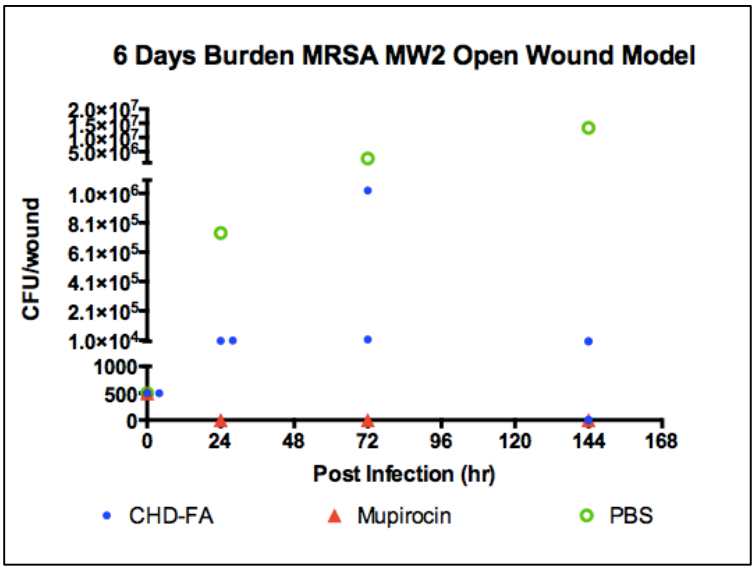
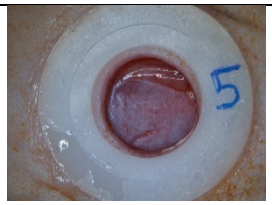
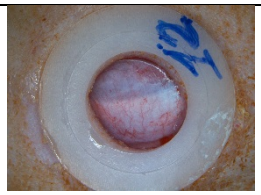
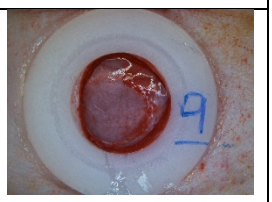
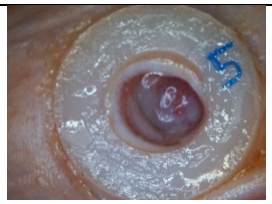
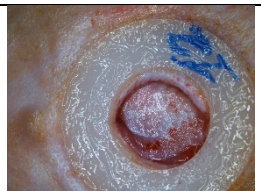
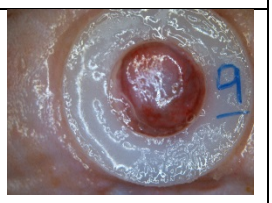
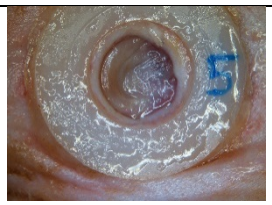
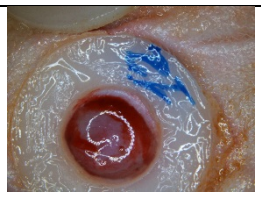
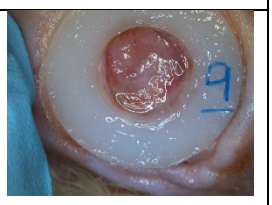
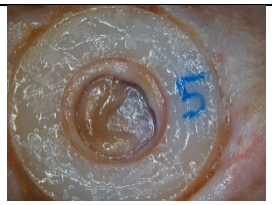
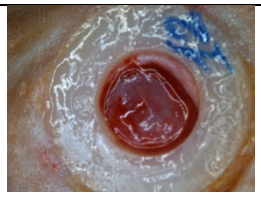
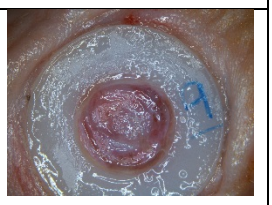
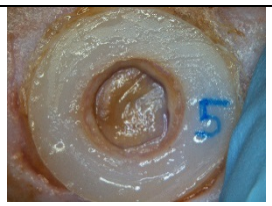
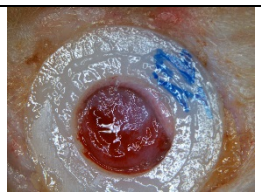
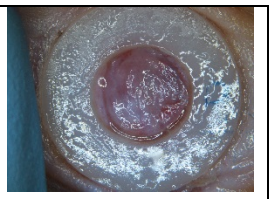
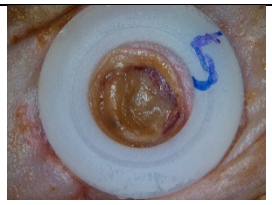
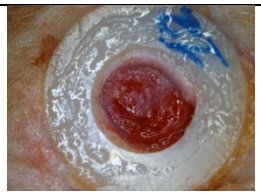
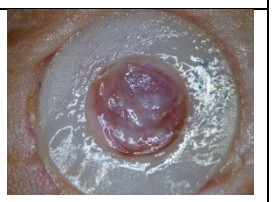
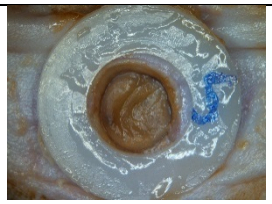
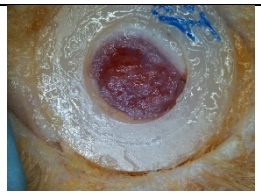


Figure 8. Cutaneous wound images of rats infected with 500 CFU of MRSA (MW2) and treated with CHD-FA at 0.5h post infection.

Day	4.6% CHD-FA	PBS	Mupirocin
0			
1			
2			
3			

4			
5			
6			

Section 8. Evaluation of CHD-FA with modified application to treat burns wounds infected with *P. aeruginosa*.

To assess the efficacy of CHD-FA with modified application against burn wounds infected with *P. aeruginosa* strain Xen5, a total of 12 rats were randomized into three treatment groups: 6 in CHD-FA (4.6%) group, 3 in PBS group, and 3 in Silver Sulfadiazine (standard of care for burn wounds) group.

P. aeruginosa strain Xen5 was inoculated in LB media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to $2x10^4$ CFU per ml for the infection. Two same size burn wounds were created on each rat, for a total of 24 wounds. The process of anesthetizing the rats and creating the burn wounds was the same as previously described in burn wound infection studies. After wound creation, rats from each treatment group were infected with 0.025 ml of the *P. aeruginosa* cell suspension at a final infection dose of 500 CFU.

A circular gauze dressing of 8mm in diameter was saturated overnight with either CHD-FA or PBS in sterile centrifuge tubes. These saturated dressings were applied to the wound site with sterile forceps twice daily starting at 30 min post inoculation**. Figure 9** shows representative images of the wounds over the course of day 0 (day of wound creation) through day 6.

Compared to PBS and the current standard of care, silver sulfadiazine treated burn wounds, less inflammation was observed in wounds treated with CHD-FA. Bacterial burden in the wounds treated with CHD-FA was significantly reduced by 3.8, 4.0, and 6.6 log relative to the untreated control at 1 day, 3 days, and 6 days post-inoculation, respectively. In comparison, silver sulfadiazine treated wounds did not have significant reduction throughout the 6-day experiment. (**Figure 10, Table 7**.)

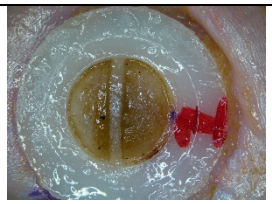
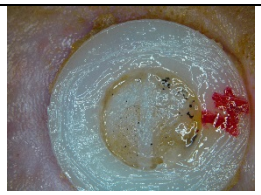
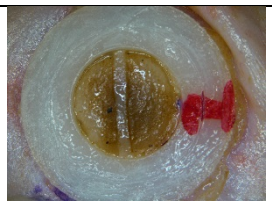
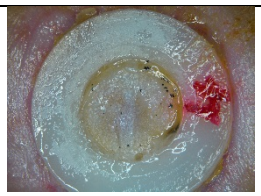
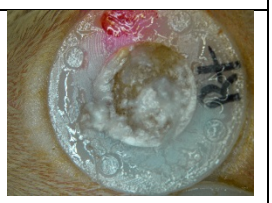
To assess wound healing, gene expression profiling was performed using a commercial PCR array (The Rat Wound Healing RT² Profiler™ PCR Array, Cat No. 330231, Qiagen), as previously described.

Similar with what was observed from the cutaneous wounds infected with *P. aeruginosa*, a panel of genes including CCL12, CCL7, CSF2, CSF3, CXCL1, CXCL11, CXCL3, CXCL5, IL10, IL1b, IL6, MMP9, PTGS2, SERPINE1, and TNF were up-regulated post infection (**Figure 11a**). The kinetic changes of these genes during the study period were compared among treatment groups (**Figure 11b**). At day 1 post inoculation, the untreated wound had extremely highly expressed inflammatory chemokines, especially CSF3, CXCL3, and IL6 compared to the CHD-FA treated wound (**Table 8, Figure 11b**), indicating a provoked inflammation on the wounds upon infection. Moreover, expression level of the key biomarker of impaired wound healing, IL6, was high throughout the experiment with 2613, 172, 79-fold increases on day 1, 3, and 6, respectively. In contrast, in the CHD-FA treated wound, the expression level remained low (59, 10, 13-fold increase on day 1, 3, and 6, respectively) throughout the experiment **(Table 8, Figure 12)**. The rapidly dampened expression of inflammatory chemokines and cytokines, especially IL-6, clearly demonstrated the accelerated wound healing of the CHD-FA treated wounds, compared to untreated controls.

Conclusion

Similar to what was observed in the *P. aeruginosa* cutaneous infection model, CHD-FA treated burn wounds had a significant bacterial burden reduction, whereas untreated wound burden remained high over the course of 6-day experiment. In conclusion, CHD-FA has demonstrated consistent potency against *P. aeruginosa* caused burn wound infections.

Figure 9. Burn wound images of rats infected with 500 CFU of *P. aeruginosa* (Xen3) and treated with CHD-FA at 0.5h post infection.

Day	4.6% CHD-FA	PBS	Silver Sulfadiazine
0			
1			
2			

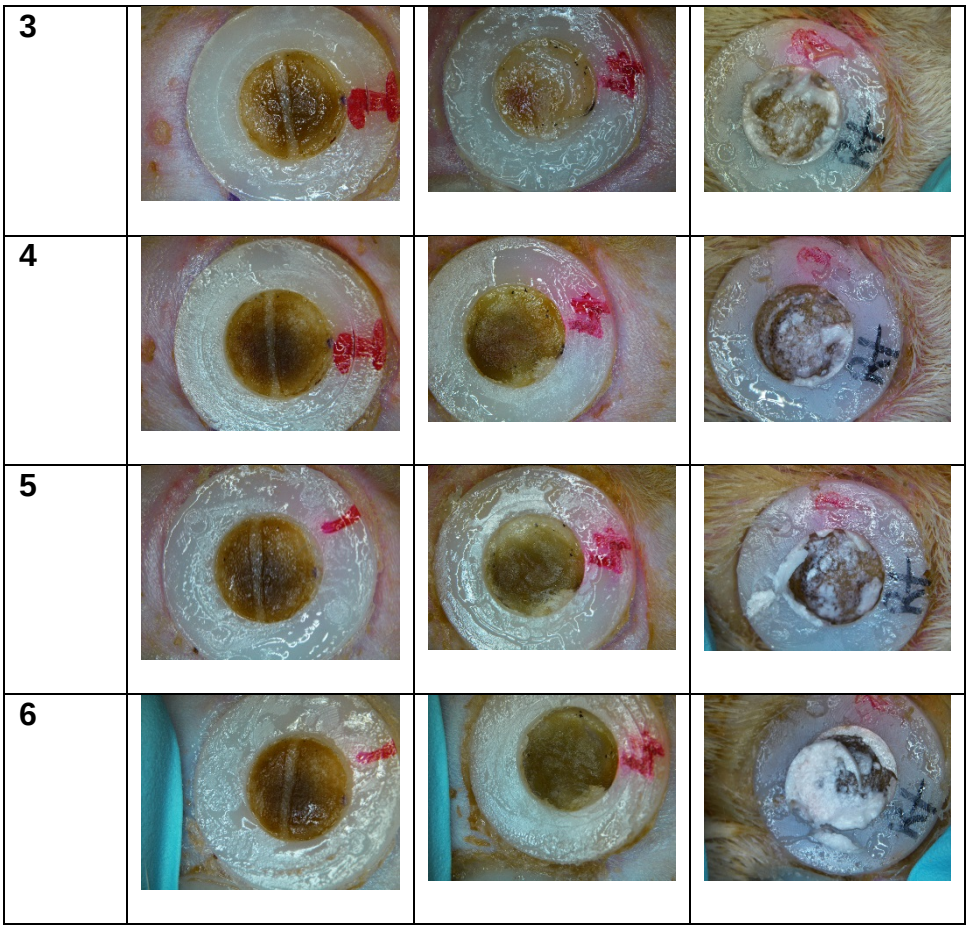


Table 7. Bacterial burdens of burn wounds infected with *P. aeruginosa* at Day 1, 3, and 6 experimental endpoints.

P. aeruginosa 500CFU	Day 1			Day 3			Day 6			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	3.8	NA	NA	4.0	NA	NA	8.0	NA	NA	*P. aeruginosa* 500 CFU
CHD-FA	Sterilized	NA	3.8	Sterilized	NA	4.0	1.4	0.0-1.4	6.6	
AB Control	3.2	NA	0.6	4.3	NA	-0.4	6.8	NA	1.2	

Figure 10. Bacterial burdens of burn wounds infected with *P. aeruginosa* vs. post-inoculation time.

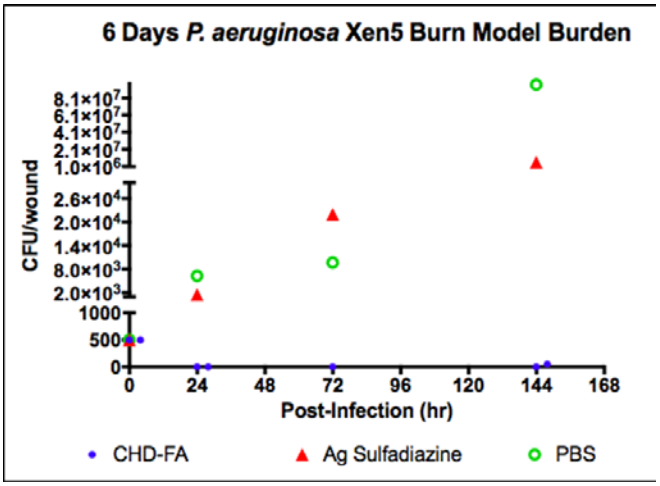


Figure 11a. Scatter plot expression profiling of host wound healing genes along the study period at day 1, 3, and 6.

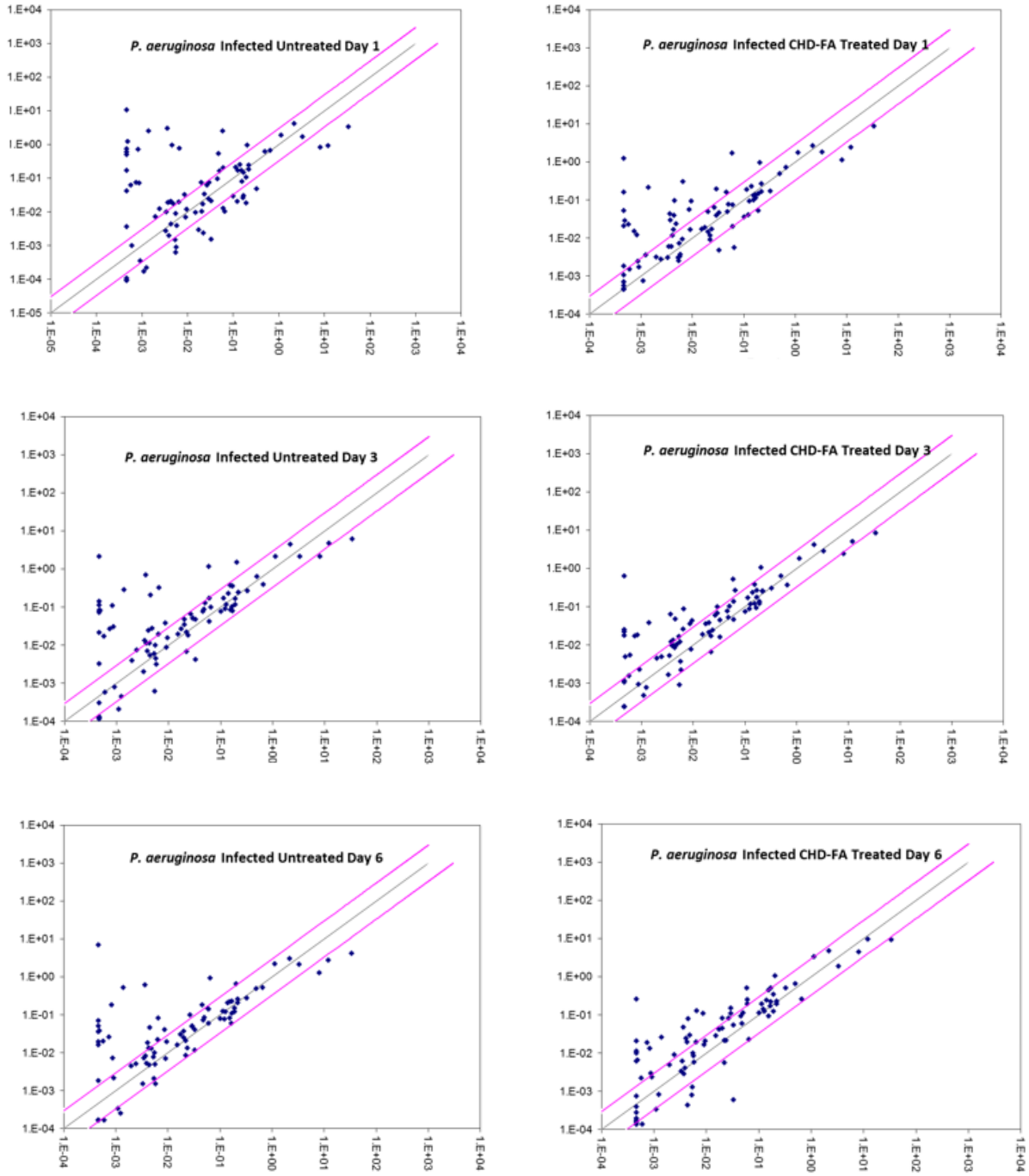


Figure 11b. Kinetic changes of representative wound healing genes

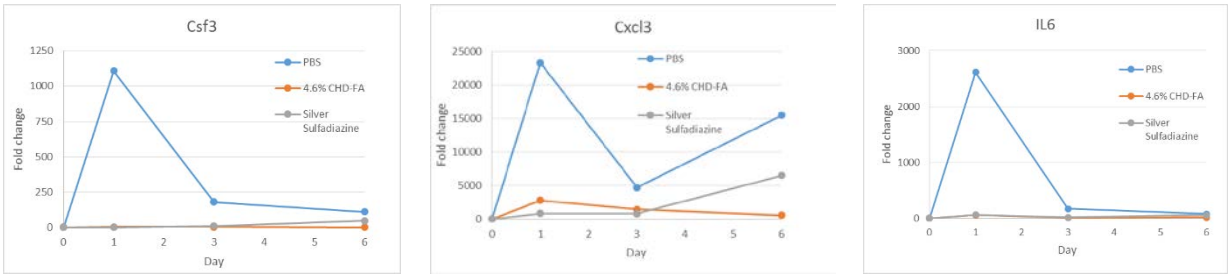
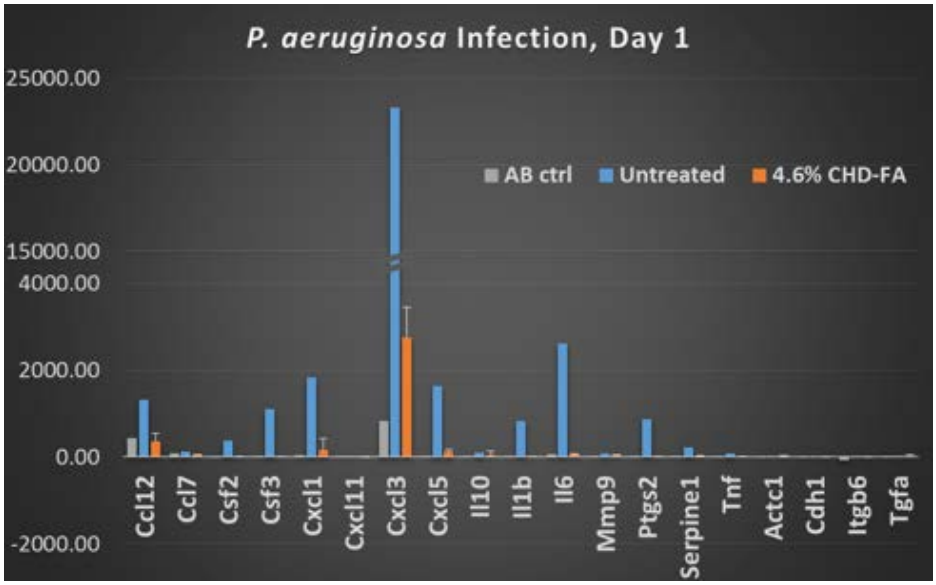
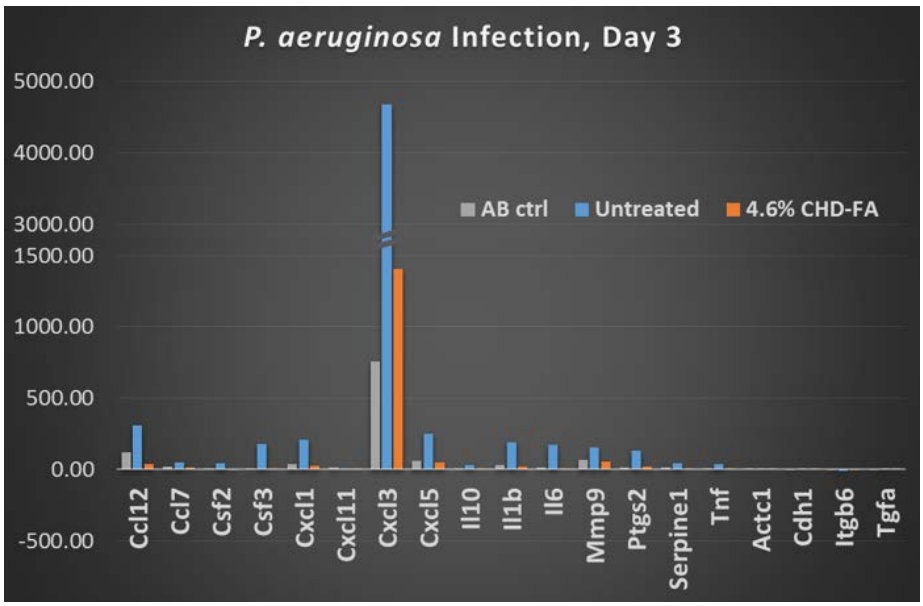


Figure 12. Fold changes of host wound healing genes treated with CHD-FA.





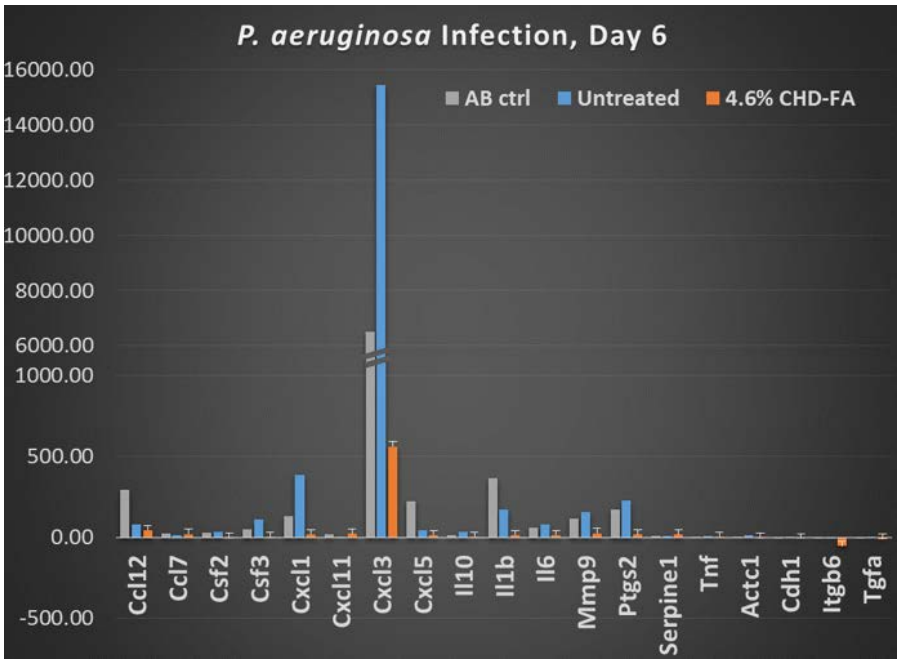


Table 8. Kinetic changes of prominently up-regulated wound healing genes among different treatment groups

Genes	AB Control				Untreated				4.6% CHD-FA			
	Day 0	Day 1	Day 3	Day 6	Day 0	Day 1	Day 3	Day 6	Day 0	Day 1	Day 3	Day 6
Ccl12	1	436.85	117.70	295.09	1	1329.81	309.54	77.76	1	349.46	39.31	44.97
Ccl7	1	72.55	19.82	24.42	1	119.59	50.18	12.56	1	47.64	13.47	19.99
Csf2	1	-1.48	1.49	27.76	1	376.63	46.66	35.41	1	1.23	-1.84	-2.30
Csf3	1	1.52	9.91	47.84	1	1106.66	180.89	110.74	1	4.08	2.30	1.65
Cxcl1	1	50.95	35.48	131.14	1	1822.88	209.24	385.61	1	157.48	28.09	19.17
Cxcl11	1	8.03	12.99	19.56	1	7.87	7.23	4.03	1	1.56	2.54	22.02
Cxcl3	1	820.86	756.92	6494.49	1	23283.17	4669.33	15457.29	1	2757.22	1405.63	560.67
Cxcl5	1	31.47	59.47	223.63	1	1631.52	247.97	42.25	1	116.48	48.40	13.32
Il10	1	9.23	6.72	15.73	1	110.43	30.46	35.29	1	40.76	2.77	3.89
Il1b	1	30.82	34.04	364.56	1	838.69	193.88	171.97	1	12.37	17.72	13.32
Il6	1	61.22	13.12	57.08	1	2613.92	172.33	79.40	1	59.26	10.43	13.75
Mmp9	1	11.20	65.75	117.78	1	91.90	158.02	154.99	1	46.01	55.99	25.21
Ptgs2	1	5.47	12.20	171.25	1	874.31	134.74	227.70	1	14.97	22.66	16.35
Serpine1	1	10.42	12.59	9.03	1	214.82	46.66	10.38	1	21.54	10.91	18.20
Tnf	1	2.36	6.75	4.96	1	82.54	34.99	8.40	1	2.80	1.07	3.45

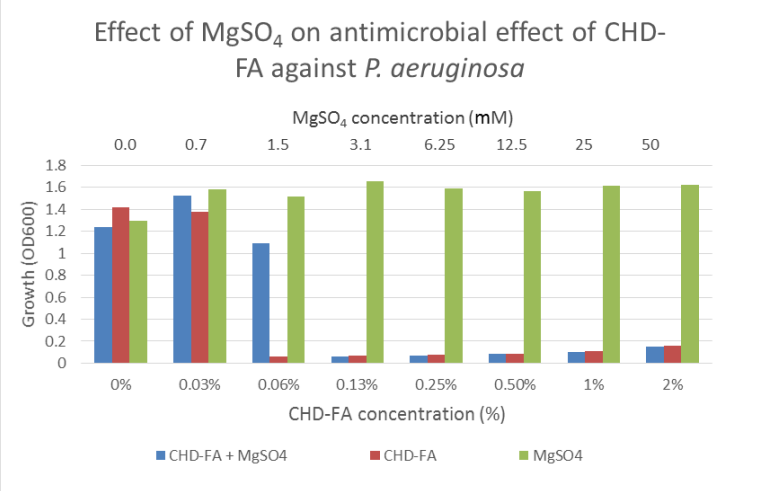
Section 9. Effects of magnesium sulfate on the antimicrobial properties of CHD-FA

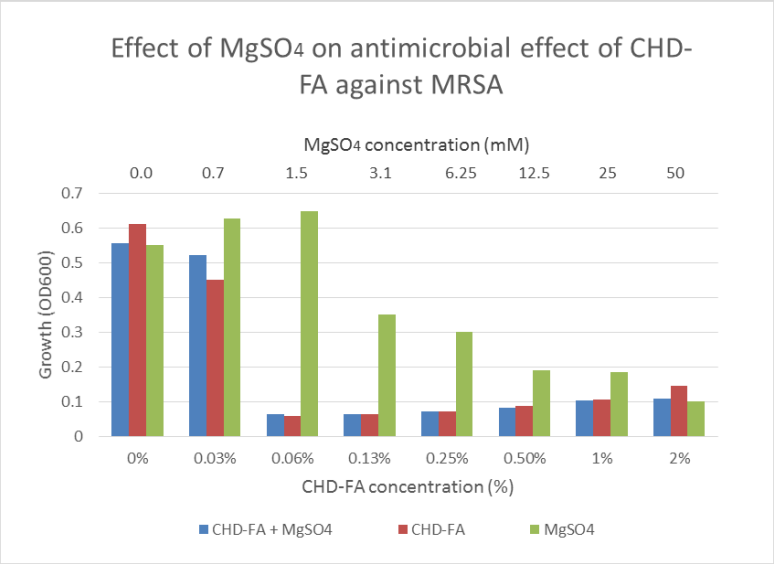
Preliminary studies suggest that CHD-FA may exert its antimicrobial behavior through direct or indirect metal starvation of cells. In the last quarterly report submitted on 4/13/16, the impact of iron on the antimicrobial efficacy of CHD-FA was investigated, but not much antimicrobial killing effect difference was observed with or without iron. In this report, the effect of magnesium on antimicrobial activity of CHD-FA was tested. The hypothesis assessed was that CHD-FA may deprive bacteria of magnesium essential for cell growth and hence impairs the ability of bacteria to survive in the host during the infection. MIC testing was performed for CHD-FA against MRSA strain MW2 and *P. aeruginosa* strain Xen5 in the presence/absence of $MgSO_4$.
CHD-FA was supplied as a 4.6% Gel (Aug 2014 batch) by Fulvimed (Pty) Ltd (A division of Fulhold Ltd.), Somerset West, South Africa. CHD-FA was stored in the dark at room temperature. A highly standardized broth-based *in vitro* susceptibility assay following the Clinical and Laboratory Standards Institute (CLSI) protocol M07-A9 “Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Eighth Edition” was used to determine the MIC values. Briefly, bacterial colonies from a freshly grown agar plate were re-suspended in CLSI recommended cation-adjusted Mueller Hinton broth for aerobic bacteria. MIC values were visually read at 48h. The MIC is defined as the lowest concentration producing a prominent decrease in turbidity compared to the drug free well. All assays were performed in duplicate.

The MIC of 4.6% CHD-FA gel against *P. aeruginosa* and MRSA was 0.13% and 0.06%, respectively in the presence of $MgSO_4$ and in the absence of $MgSO_4$ at 48h. $MgSO_4$ alone inhibited growth of MRSA but failed to inhibit growth of *P. aeruginosa*.

Overall, there was no significant change in the MIC against *P. aeruginosa* or MRSA treated with CHD-FA in the presence of excess $MgSO_4$. These preliminary results suggest that magnesium has no impact on antimicrobial potency of CHD-FA and divalent cation chelation is not a likely MOA (See **Figure 13**.)

Figure 13. MIC of *P. aeruginosa* Xen5 and MRSA MW2 to CHD-FA in the presence of $MgSO_4$





Section 10. Evaluation of CHD-FA with modified application to treat burn wounds infected with MRSA.

To assess the efficacy of CHD-FA with modified application against burn wounds infected with MRSA strain MW2, a total of 12 rats were randomized into three treatment groups: 6 in CHD-FA (4.6%) group, 3 in PBS group, and 3 in Mupirocin group.

MRSA strain MW2 was inoculated in LB media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to $2x10^4$ CFU per ml for the infection. Two same size burn wounds were created on each rat, for a total of 24 wounds. The process of anesthetizing the rats and creating the burn wounds was the same as previously described in burn wound infection studies. After wound creation, rats from each treatment group were infected with 0.025 ml of the MW2 cell suspension at a final infection dose of 500 CFU.

A circular gauze dressing of 8mm in diameter was saturated overnight with either CHD-FA or PBS in sterile centrifuge tubes. These saturated dressings were applied to the wound site with sterile forceps twice daily starting at 30 min post inoculation**. Figure 14** shows representative images of the wounds over the course of day 0 (day of wound creation) through day 6.

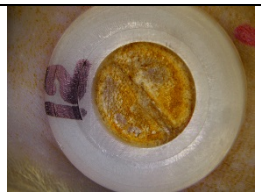
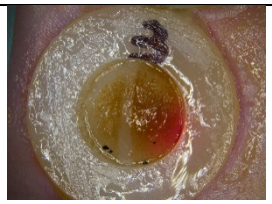
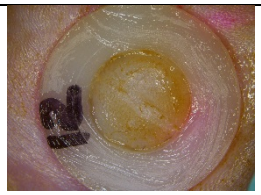
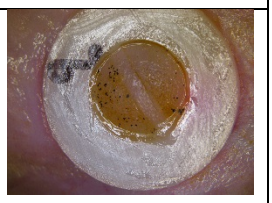
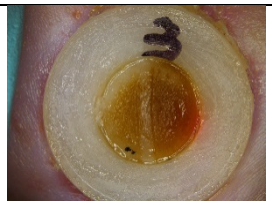
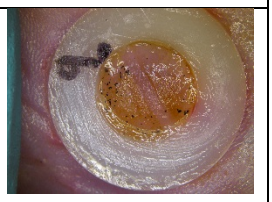
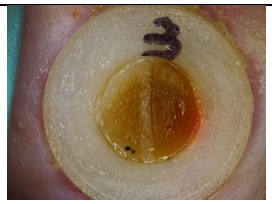
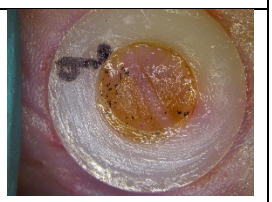
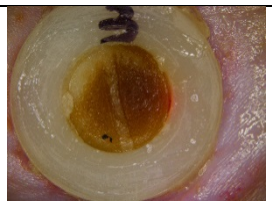
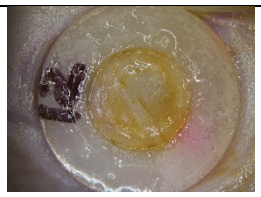
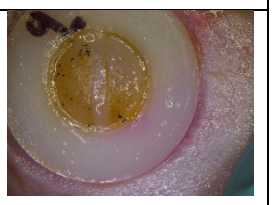
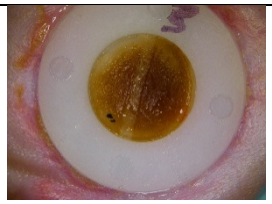
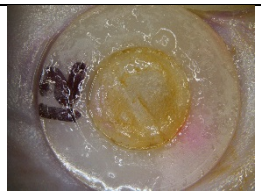
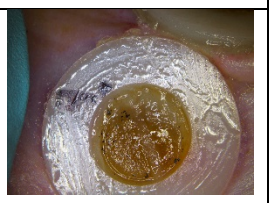
The bacterial burden assessment of the wounds at varying time points showed a 5.8 log burden reduction in the wounds for the CHD-FA treated group relative to the untreated controls at 24h. However, recovery of the microbial burden was observed in CHD-FA treated group after 48h post-inoculation **(Table 9, and Figure 15)**. Therefore, no significant burden reduction was observed from CHD-FA treated wounds compared to the negative control treated wounds from day 3 to day 6 experimental endpoints. The future experiments will be focused on analyzing properties of CHD-FA that makes it more effective against gram negative bacteria than gram positive bacteria. Other cutaneous and burn wound infection models with gram negative organisms such as *Acinetobacter baumanii* will also be performed this year.

Conclusion

Twice daily application of saturated CHD-FA gauze was still not sufficient to control infections from MRSA in a 6-day burn wound model. No significant changes in microbial burden reduction were

observed in this trial as observed previously in the MRSA cutaneous wound infection model. CHD-FA may be not have as potent antimicrobial properties against burn infections caused by Gram positive bacteria as that against infections caused by Gram negative bacteria.

Figure 14. Burn wound images of rats infected with 500 CFU of *P. aeruginosa* (Xen3) and treated with CHD-FA at 0.5h post infection.

Day	4.6% CHD-FA	PBS	Mupirocin
0			
1			
2			
3			
4			
5			

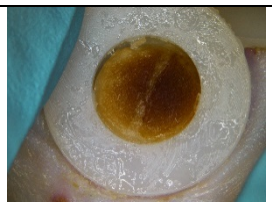
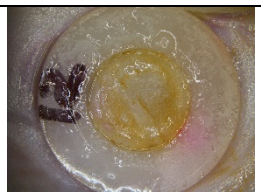
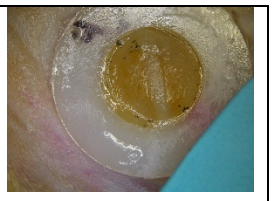
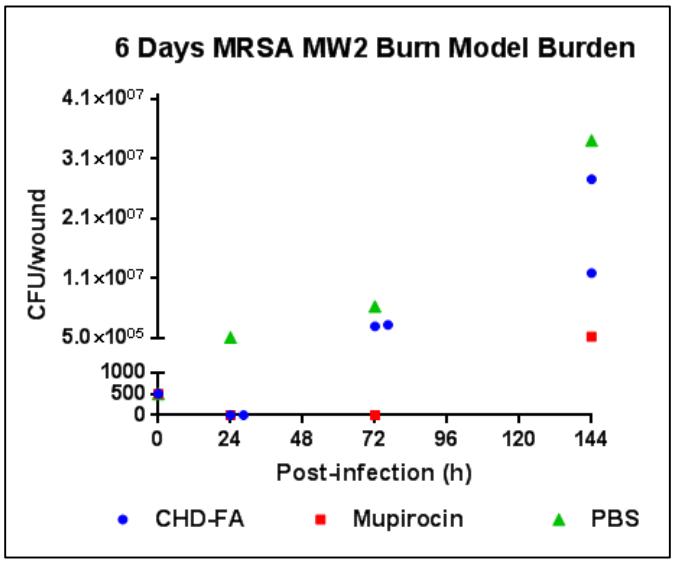
6			

Table 9. Bacterial burdens of burn wounds infected with MRSA at Day 1, 3, and 6 experimental endpoints.

MRSA 500CFU	Day 1			Day 3			Day 6			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	5.8	NA	NA	6.8	NA	NA	7.5	NA	NA	MRSA 500 CFU 0.5h Tx
CHD-FA	Sterilized	NA	5.8	6.4	NA	0.4	7.2	7.1-7.4	0.3	
AB Control	Sterilized	NA	5.8	Sterilized	NA	6.8	5.8	NA	1.7	

Figure 15. Bacterial burdens of burn wounds infected with MRSA vs. post-inoculation time.



Section 11. Establishing the wound infection in rats with *Acinetobacter baumannii* strain 5075

To establish the cutaneous wound model in rats with the multi-resistant *Acinetobacter baumannii* strain 5075, 9 rats were used for infection. As with the previously reported virulence assessment studies, 9 rats were randomized into three infection groups (3 rats per group). *A. baumannii* strain 5075 was inoculated in LB media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to 500, 5000, and $2.5x10^4$ colony forming units (CFU) per ml for the infection. Two wounds were created on each rat, for a total of eighteen wounds. The process of anesthetizing the rats and creating the wounds was the same as described above in the section on establishing the wound model. After wound creation, rats from each challenge group were infected with 0.05 ml of the cell suspension with corresponding doses. The final infection doses for the rats were 500, 5000, and $2.5x10^4$, respectively.

In all the wounds infected with *A. baumannii,* the discharge was observed throughout the 3-day experiment but was more pronounced in the higher infection doses. **(Figure 16)**.

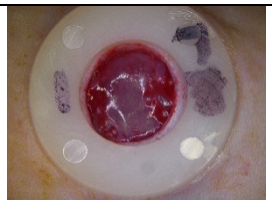
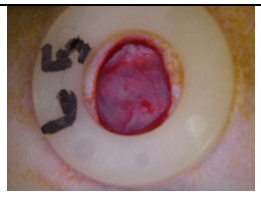
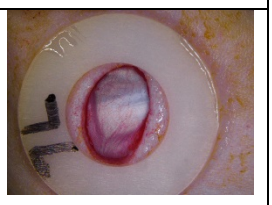
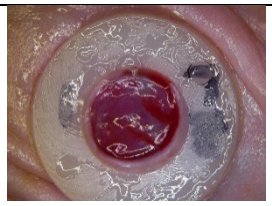
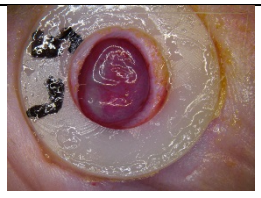
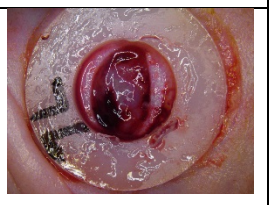
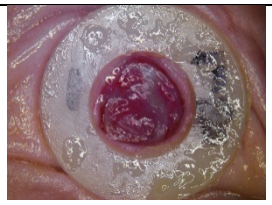
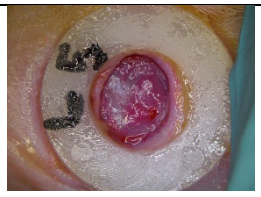
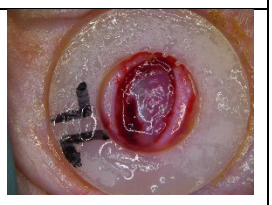
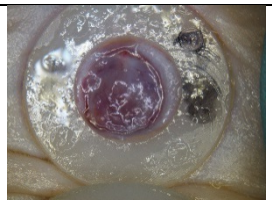
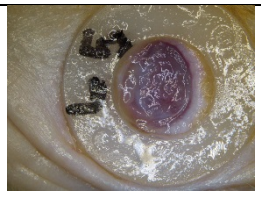
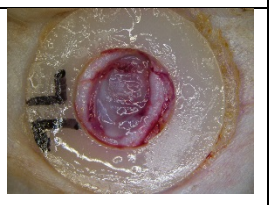
Circular gauze dressing of 8mm in diameter was saturated overnight with PBS in sterile centrifuge tubes. These saturated dressings were applied to the wound site with sterile forceps twice daily

starting at 30 min post inoculation**. Figure 16** shows representative images of the wounds over the course of day 0 (day of wound creation) through day 3. The discharge of the wounds was observed at various infection doses throughout the 3-day experiment but was more pronounced in the higher infection doses. Initiation of scab formation was not observed throughout the study. There were no observable signs of sepsis such as lethargy or recumbency as a result of infection in any of the rats. The bacterial burdens of the wounds were greatly increased by 6 logs on day 1 experimental time point, and by 7 logs on day 2 and day 3 experimental time points. Interestingly, the burdens of all infection doses did not differ significantly.

Conclusion

Based on the virulence study, a 5000 CFU infection dose will be used for all downstream *A. baumannii* cutaneous wound infections.

Figure 16. Cutaneous wounds in rats with *A. baumannii* (5075) infection.

Day	500 CFU	5000 CFU	2.5x10^4 CFU
0			
1			
2			
3			

Key Research Accomplishments

- **Obtained histopathological analyses of burn wounds from rats infected with *P. aeruginosa* and treated with CHD-FA**
- **Performed host gene expression profiling for *P. aeruginosa* and MRSA infected burn wounds, respectively, and demonstrated that CHD-FA blocks cellular inflammation and improves wound healing**
- **Demonstrated that CHD-FA saturated gauze significantly reduced microbial burdens compared to the untreated control groups in 6 days cutaneous and burn wounds infected with *P. aeruginosa***
- **Established a reproducible cutaneous wound model in rats for *Acinetobacter baumannii* that can be used to assess CHD-FA to treat wound infections**

Reportable Outcomes

In addition to the previous poster presentation (**CHD-FA is a highly effective topical broad-spectrum antimicrobial for drug-resistant wound infections**) at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC 2014, Washington DC) and the oral presentation entitled "**Evaluation of Carbohydrate Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug Resistant Wound Infections**" at the Military Health System Research Symposium (MHSRS) 2014, "**CHD-FA is a highly promising topical broad-spectrum antimicrobial for drug-resistant wound infections"** at the MHSRS 2016, and "**Evaluation of Carbohydrate Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug Resistant Wound Infections**" at the JPC 2016. We have published an original article "Carbohydrate-derived fulvic acid is a highly promising topical agent to enhance healing of wounds infected with drug-resistant pathogens" on the Journal of Trauma and Acute Care Surgery in October 2015. During the past year, we have successfully established and optimized the burn wound model. To resolve the problem of diminished *in vivo* antimicrobial activities of the original CHD-FA formulation, we have made great efforts in prompting CHD-FA formulation modification as well as in evaluating new formulations comprehensively and critically. By far, the twice daily treatment CHD-FA saturated gauze is the most promising method, by which a significantly increased antimicrobial activity against *P. aeruginosa* infections was observed in the 6-day studies on both cutaneous and burn wound model.

Dr. Perlin was awarded a NIH Research Program – Cooperative Agreements (U19) grant (RFA-AI-12-044) that resulted in PHRI-Rutgers University being designated as one of the NIAID Centers for Excellence in Translational Research (CETR) to develop novel leads against drug resistant pathogens. The wound model was a significant component in the assessment of novel lead compounds against drug resistant bacterial pathogens.

Conclusion

In this fourth annual report, treatment efficacy of CHD-FA with modified application against *P. aeruginosa* and MRSA infected cutaneous and burn wounds was assessed in the 6-day study, respectively. In the cutaneous and burn wounds infected with *P. aeruginosa*, twice daily application of CHD-FA saturated dressing resulted in a rapid microbial clearance from wounds throughout the 6-day study. Host gene expression profiling analyses also suggested the twice daily CHD-FA treatment led to faster and better balanced inflammatory response and wound healing process at the cellular level. Promising, but to a lesser extent *in vivo* antimicrobial efficacy was also observed in the cutaneous and burn wounds infected with MRSA. The rapidly reduced microbial burden upon CHD-FA application restored from day 3 till day 6 in the cutaneous wound infection model. Although we have previously confirmed the broad-spectrum activity of CHD-FA *in vitro*, CHD-FA may be less active against Gram-positive pathogens *in vivo*. The exact molecular mechanisms of the antibiotic activity of CHD-FA are still not clear, and will be further investigated to address the discrepancy in its activity against Gram-positive and Gram-negative pathogens in our future work. During the next quarter and throughout next year, we will perform studies with a modified application method (dressing) of CHD-FA on other pathogens listed in the statement of work. Overall, we have made strong progress in the fourth year. We believe that the CHD-FA combined with the modified application method will be effective in treating wounds infected with clinically important drug resistant pathogens and demonstrate its potency for preventing wound infections and promote healing.

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Appendices

Updated Quad Chart

Evaluation of Carbohydrate Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug Resistant Wound Infections

Insert ERMS/Log Number and Task Title Here

W81XWH-11-DMRDP-MID-ARA

PI: David S. Perlin Org: New Jersey Medical School-Rutgers, The State University of New Jersey Award Amount: $1,530,400

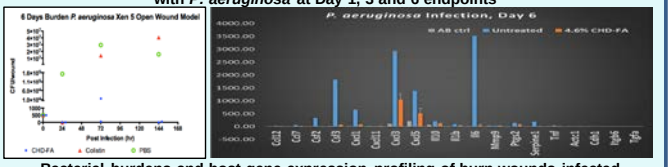
Study/Product Aim(s)

- **Assess *in vitro* susceptibility of CHD-FA against multi-drug resistant bacteria and fungal pathogens**
- **Evaluate efficacy of topical application of CHD-FA on small animal models of wound infection with a wide variety of bacterial and fungal pathogens.**

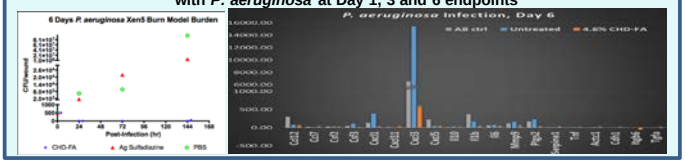
Approach

Establish minimum inhibitory concentrations (MIC_{50} and MIC_{90}) for CHD-FA against large collections of clinical isolates representing wound-associated drug resistant bacteria and fungi. Rat models of wound infection (open, and burn model) will be established with healthy animals using drug resistant bacterial and fungal pathogens. Increasing doses of CHD-FA in topical form will be used to assess relative efficacy.

Bacterial burdens and host gene expression profiling of cutaneous wounds infected with *P. aeruginosa* at Day 1, 3 and 6 endpoints



Bacterial burdens and host gene expression profiling of burn wounds infected with *P. aeruginosa* at Day 1, 3 and 6 endpoints



CHD-FA gel (4.6%) formulation with the modified dressing application method twice a day with saturated gauze applied to the wound site resulted in a rapid wound sterilization.

Timeline and Cost

Activities CY	12	13	14	15
Establish MIC_{90}S for CHD-FA with clinical isolates of major drug resistant pathogens				
Assess CHD-FA in animal models of wound infection for major pathogens				
in vivo evaluation of CHD-FA in open, and burn models.				
Histopathological analysis and gene expression profiling.				
Estimated Budget ($K)	**$400**	**$600**	**$500**	**$400**

Updated: October 29, 2016

Goals/Milestones

CY12 Goal – Establish (MIC_{50} and MIC_{90}) for CHD-FA

☑ *in vitro* efficacy of CHD-FA against drug resistant bacteria.

CY13 Goals – Complete *in vitro* susceptibility testing and establish cutaneous wound model in rats.

☑demonstrated the potent antimicrobial activity of CHD-FA against a broad range of drug resistant bacteria and fungi.

CY14 Goal – Establish open/ burn wound infection model in rats using drug resistant bacterial and fungal pathogens following treatment with CHD-FA in topical form.

☑ established the cutaneous wound model in rats with major pathogens.

CY15 Goal – *in vivo* evaluation of CHD-FA in open and burn models in rats.

☑ established the burn wound model in rats with MRSA, *P. aeruginosa* & *K. pneumoniae* following treatment with CHD-FA-Zn in topical form.

☑ histopathological analyses and host gene expression profiling to assess burn wound healing.

☑ *in vivo* evaluation of CHD-FA with modified dressing application method to treat twice a day cutaneous and burn wounds infected with key drug resistant pathogens

☑ **Comments/Challenges/Issues/Concerns**

- application of a dressing over the wound may induce bleeding from changing dressing due to the adherent nature of the gauze and potentially delay the wound healing.
- **Budget Expenditure to Date**

Projected Expenditure:$1,530,400

Actual Expenditure: $1,512,933

"Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections."

Quarterly Technical Progress Report (Q13.01.2016)

Performance period: 10/29/2015 through 12/13/2016.

Date of report: January 13, 2016

Public Health Research Institute
New Jersey Medical School, Rutgers, The State University of New Jersey
225 Warren Street
Newark, NJ 07103

Award Recipient/Principal Investigator: **David S. Perlin, Ph.D.**
Tel. 973-854-3200

Grants Officer: **Alla Rabinovich, M.B.A.**
Tel. 973-854-3115

"Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections."

Quarterly Technical Progress Report (Q14.4.2016)

Performance period: 12/13/2015 through 3/31/2016.

Date of report: April 13, 2016

Public Health Research Institute
New Jersey Medical School, Rutgers, The State University of New Jersey
225 Warren Street
Newark, NJ 07103

Award Recipient/Principal Investigator: **David S. Perlin, Ph.D.**
Tel. 973-854-3200

Grants Officer: **Alla Rabinovich, M.B.A.**
Tel. 973-854-3115

"Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections."

Quarterly Technical Progress Report (Q14.7.2016)

Performance period: 4/1/2016 through 7/1/2016.

Date of report: July 14, 2016

Public Health Research Institute
New Jersey Medical School, Rutgers, The State University of New Jersey
225 Warren Street
Newark, NJ 07103

Award Recipient/Principal Investigator: **David S. Perlin, Ph.D.**
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